FERMENTATION OF PURE SUGARS FOUND IN WASTE SULPHITE LIQUOR

by

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ABSTRACT

In this work, pure solutions of the hexoses found in softwood were fermented. The solutions consisted of 33.3 gm sugar per liter. The study consisted of three parts. The purpose of the first part was to test the newly assembled equipment by verifying results published by Anderson [19]. In this part a method was also developed to calibrate dissolved oxygen probes. In the second part, the glucose fermentation by Saccharomyces Cerevisiae was studied in detail using the factorial design method. Three variables were investigated: temperature, pH and rpm, for respective intervals of 27-32°C, 4.0-6.0 and 500-700. With the results from above and those obtained by Aiyar and Luedeking [26] we found that the highest production of ethanol was achieved for the following conditions:

- temperature: 32°C
- pH : 5.0
- rpm: 700

In the third part, these conditions were also applied to the fermentations of galactose and mannose, which form about 85% of the hexose concentration of softwoods.

Finally, a model was proposed to describe the galactose and mannose fermentations, which did not follow the Michaelis-Menten model nor the Kono-Asai model.

ACKNOWLEDGEMENTS

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INTRODUCTION

The carbohydrates in spent liquor from the acid sulphite pulping process have caused problems during the entire period that sulphite pulp has been produced commercially. It is these wood sugars and their degradation products which chiefly account for the biochemical oxygen demand (B. O. D.) of this pulp mill effluent when discharged to receiving waters. The competitive status of the sulphite pulping industry is seriously threatened by the prospect of forced installation of costly pollution control equipment and investigation of processes for the profitable utilization of these wood sugars is still the subject of an enormous effort in pulping research centers.

The availability in quantity is a first step along the road to economic utilization. The average sized sulphite pulp mill has some 15 to 30 tons of sugar available from its daily production, an adequate basis for byproduct recovery schemes. The hydrolytic reactions which occur during the pulping of wood by the acid sulphite process cause the polysaccharides originally present in the wood substance to break down in various degrees. The final spent liquor is derived from controlled rates and degrees of pulping which produce different grades and types of cellulose fiber from one or more of the numerous kinds of wood utilized. These spent liquors can therefore be expected to contain a variety of carbohydrates including typical wood derived hexose and pentose sugars, degradation products of these sugars, and small amounts of oligosaccharides. Free wood sugars in solution are the predominant carbohydrates in most spent sulphite liquors, and usually range from 15 to 20% of the total dissolved solids. Table 1, taken from Wiley et al. [1] provides analytical information on the composisition of the dissolved solids of representative spent sulphite liquors produced from softwoods and hardwoods.

Table 1 Components of spent sulphite liquor

	Softwood, %	Hardwood, %
Lignosulphonates	55	55
Hexoses	14	4
Pentoses	6	16
Sugar acids and residues	12	12
Resins and extractives	3	3
Ash	10	10
	100	100

In considering utilization of the carbohydrates, it is of particular interest to note the differences in the relative content of hexose and pentose sugar in the liquor from these two types of wood. Most of the sugars in softwood liquor are hexoses, while in hardwood liquor the pentoses are predominent. This relationship has an important effect upon the products which can be produced from the pulping of the two different types of wood. Table 1 also shows that there is an appreciable content of sugar degradation products about which much yet remains to be learned.

The individual sugar components, typical of the spent liquors produced from softwood and hardwood liquors are shown in Table 2. Mannose is the predominant hexose sugar in liquors derived from softwoods, while xylose is the predominant pentose sugar from the hardwood spent liquors. Glucose is found in minor quantities in both types of spent liquor. Galactose, available particularly from softwoods, and arabinose, available in smaller quantities, are the two remaining wood sugars found in spent sulphite liquor.

Table 2 Wood sugars in spent sulphite liquor

	Softwood %	Hardwood %
Hexoses		
Mannose	8	2
Galactose	4	0.5
Glucose	2	1.5
	14	4
Pentoses		
Xylose	5	15.5
Arabinose	1	0.5
	6	16

The scope of this work included the batchwise fermentation of glucose, based on a factorial design matrix. Temperature, pH and rpm variations were investigated. Also attempted in this work was the fermentation of galactose and mannose, based on the results obtained from the glucose fermentations.

LITERATURE SURVEY

1. Chemical Methods

Wiley et al.[1] mentioned a number of methods of separating sugars and lignosulphonates in addition to fermentation. Methods such as ion exclusion or ion retardation; precipitation of the lignosulphonates in sulphite liquor with strongly alkaline aqueous solutions of lime; treatment of the dry sulphite liquor solids with certain organic solvents in the presence of an acid catalyst, which results in the formation of sugar derivatives which are soluble in the solvent and can be separated from the insoluble lignosulphonates. Mokuzai Kenkyu[2] recovered the sugars present in the sulphite waste liquor by dialysis and ion exchange resin in his study to relate the BOD to the sugars present. Boggs[3] isolated four of the five main sugars in sprucewood spent sulphite liquor as crystalline compounds. He further identified them by reduction to the corresponding crystalline polyols. He altered the properties of the reducing sugars by conversion to their acetone-soluble di-0-isopropylidene derivatives. The main portion of the nonsugars remained insoluble and was filtered off. Di-0-isopropylidene D-mannose is sparingly soluble in water, from which it was crystallized. The di-0-isopropylidene pentoses were separated from the hexose derivatives by two methods:

1. High vacuum distillation, wherein the pentose derivatives were more readily volatilized than the hexose derivatives.
2. Exhaustive steam distillation, wherein the pentose derivatives were volatile and the hexose derivatives were not.

The resulting fractions were further fractionated into acid-sensitive derivatives and acid resistant derivatives. Acid hydrolysis, to remove the remaining isopropylidene groups yielded the free sugars.

2. Biological Methods

Traditionally however, studies have been centered on fermentation methods. One advantage of fermentation is that the sugars are converted to products which are more easily recovered from the dilute solution. Some of the products which can be produced from sulphite liquor by fermentation are fumaric acid, butanol and acetone.

2.1 Fumaric acid fermentation

A. Romano [4] undertook a study to investigate the feasibility of utilizing a fungus fermentation to convert sugars in spent sulphite liquor to fumaric acid. The fungus used was Rhizopus cryzac NRRL 1526 obtained from Northern Regional Research Laboratory, Peoria, Ill. All experiments reported were carried out with calcium base spent sulphite liquor which had been concentrated to 57% solids. Production of fumaric acid was demonstrated in both growth and replacement phases. Concentrations of nutrient to be added to spent sulphite liquor were determined by experiment and found to be critical for the production of fumaric acid. Based on the total amount of sugar available, over-all fumaric acid yields of 40% by weight were obtained.

2.2 B.O.D. Reduction

Amberg and Cormack [5] presented some data obtained from laboratory bench scale operations as well as some data from a semi-pilot plant for the biological treatment of spent sulphite liquor. They modified an activated sludge process using an aerobic fermentation process, capable of reducing 80 to 90% of the BOD of spent sulphite liquor by employing a mixed bacterial culture. In their laboratory bench-scale

experiments, they introduced new ammonia-base spent sulphite liquor into an aeration tank, where it was aerated at 35°C in the presence of bacterial cells. The only nutrient required was phosphorus. The mixed liquor was discharged by gravity to a conical settling basin where the cells were separated from the supernatant liquor. The effluent was discharged to the sewer. The settled cells were pumped back to the aeration chamber, and once each day the excess cells which accumulated in the clarifier were withdrawn to maintain a constant concentration of cells in the aeration unit. Microscopic examination of the organisms present in the aeration tank indicated that the predominant culture consisted of Gram-negative rods of the Pseudomonas group. Excellent results were reported up to a waste concentration of 8.5% total solids. Analyses of the bacterial cells produced showed that they contained 66 to 70% protein and the material could be utilized as a high-protein animal feed. The semi-pilot plant experiments showed further that considerable savings were affected by SO_2 removal. This resulted in a 50% reduction in the quantity of neutralization chemicals used.

2.3 Butanol and acetone production

Schoedler [6] reported results obtained by fermenting spent sulphite liquor to produce butanol and acetone using butylogenic bacteria. He found it to be advantageous to utilize spent sulphite liquor in preference to maize or molasses. He obtained maximum yields by using liquors diluted with accumulated wash waters to contain 2.5-3% sugars at an optimum temperature of 30-37°C. The initial pH value of 6.5-6.8 decreased to 5.6-5.8 during the first 12 hours, but

increased to 6 and higher values during further fermentation. Furfural and HCO_2H present in small amounts did not inhibit fermentation, H_2SO_4 and AcOH, however inhibited bacterial growth. Lignosulfonic acid did not affect the course of fermentation and thus did not need to be precipitated.

2.4 Yeast production

In 1959, Kryuchkova and Korotchenko [7] studied the behaviour of different strains of yeast in fermenting spent sulphite liquor to produce baker's yeast. They carried out experiments with three strains of Saccharomyces Cerevisiae Saccharomyces (I) Tomskaya 7, L-33, KKS-1 and CKhS-5. They kept the temperature in the tanks at 30-32°C and bubbled air through at a rate of 27-29 l/min. They obtained a very high yield when CKhS-5 was blended with SK-4. Thus processed yeast leavened dough in 35-40 min. and gave tasty bread. Three years later, Schmidt and Winter [8] worked on the production of fodder yeast from sulphite black liquor, and in 1964, Stefan [9] achieved a production of 5.58 tons of fodder yeast per day in a fermentor described as follows: height 14.5 m; diameter 6.4 m; volume 450 m^3. His unit was fed with a liquor of 20-22 g/l sugar, it fermented for 5 hours and necessitated 3500-4500 m^3 of air per hour. In 1965, Amberg [10] discussed a diversity of solutions to the sulfite problem, however, as he noted, by-products recovery is the only one which completely removes the wood sugars and lignin.

2.5 Volatile acid production

In a more recent publication, Amberg [11] undertook a study with his co-workers to determine if volatile acids could be produced by fermentation of the sugars in spent

sulphite liquor. They fermented steam-stripped calcium base spent sulphite liquor on a continuous flow basis using a mixed bacterial culture. E. Coli was the major organism involved; species of streptococci were also detected. Experiments were run at 37°C and at 55°C. It was found that acid yield could be increased almost three-fold by reducing the fermentation temperature to 37°C. The optimum pH was found to be 7 to 7.5. Alcohol production of about 2 g/l was found to be a wasteful side reaction which utilized sugars that otherwise would be fermented to volatile acids. Therefore volatile acid production was stimulated by adding small amounts of air, which decreased the alcohol production with consequent increase in volatile acid yields. Acetic acid yield was reported to be 155 kg/metric ton of spent sulphite solids, at volumetric waste retentions ranging from 20 to 30 hr, and pentose-sugars remaining in alcohol-fermented spent sulphite liquor could be fermented to yield about 81.5 kg of volatile acids per ton of spent sulphite solids. The volatile acid composition was determined to be 83% acetic acid, 9.3% butyric acid, 4.8% formic acid and 2.9% propionic acid. Optimum yield was obtained from fermenting stream-stripped spent sulphite liquor containing 9 to 11% total solids. The mixed bacterial flora responsible for the fermentation also produced considerable quantities of vitamin B12. When aeration was used, 24 μg/g of cells was obtained compared to 0.66 μg/g of cells under anaerobic conditions. The process they proposed for recovery of the volatile acids consists of the following steps:

1. Evaporation of the fermented liquor to 40-50% solids.
2. Addition of sulfuric acid to adjust pH to 4.0.
3. Solvent extraction with methyl-ethyl-ketone.

4. Dehydration and recovery of solvent by azeotropic distillation.

5. Refining of 95% acetic acid to technical grade glacial acetic acid.

2.6 Mushroom production

In 1973, Kosaric et al. [12] looked into the possibility of a submerged cultivation of edible mushrooms. Four species of morel mushroom were investigated: Morchella spp., Morchella crassipes, Morchella deliciosa and Morchella esculenta. Studies of the optimal growth conditions in terms of pH and substrate concentration on the yield of biomass yielded the following results:

1. The optimal initial pH for the different morel species was found to be between 5.0 and 7.0.

2. The highest yield (83.4% based on utilized carbohydrate) was obtained when the NH_3 - waste sulphite liquor was diluted (1:5 v/v) and used for growth of Morchella crassipes.

3. In all cases, growth was found to be completely inhibited by calcium.

4. Morel mushroom mycelium contained protein of 25.7 to 48% on a dry basis, with an acceptable spectrum of essential amino acids, except for the levels of methionine and isoleucine.

One advantage in comparison with other biotreatment systems is the fact that all the grown pellets settle down in less than two hours.

It was also found that sterilization of the culture medium is not necessary if the waste sulphite liquor is pumped directly from the digesters to the fermenters, since the digesters usually operate at 160°C and 7 atm for 6 to 12 hours. It was finally observed that Morchella esculenta is able to take up both hexoses and pentoses.

2.7 Ethanol production

Another very important commercial product is ethyl alcohol. In the ethyl alcohol fermentation by Saccharomyces yeast, only the hexoses are utilized, so that a hardwood liquor in which pentoses predominate would not be suitable. Since raw sulphite liquor may contain enough sulphur dioxide to inhibit yeast growth, it is usually necessary to reduce the SO_2 concentration by stripping or neutralization before fermentation. Figure 1 shows the outline of an ethyl alcohol process.

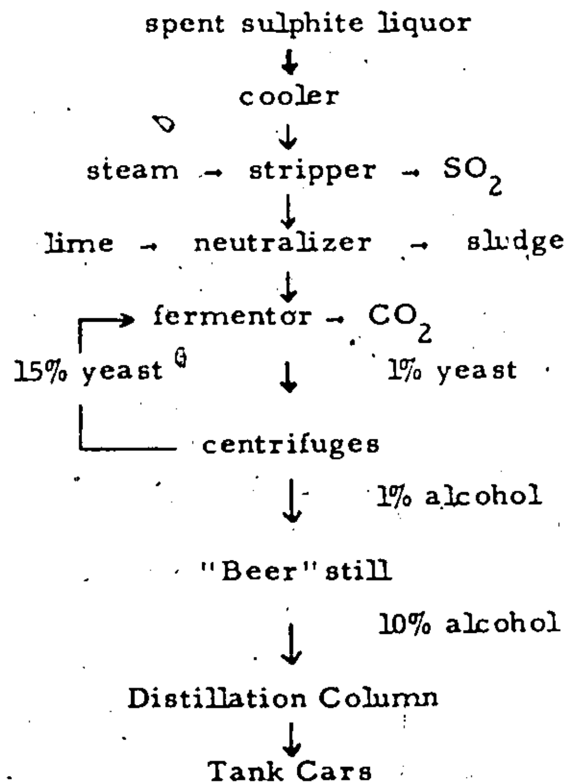

Figure 1 Ethyl alcohol from spent sulphite liquor

The production of ethyl alcohol from sulphite waste liquor has been described by Nomura [13], Prescott and Dunn [14], Gasida [15] and others. Even though the production of ethyl alcohol or yeast alone is not economical, the over-all fermentation process has additional credits which improve the picture considerably. In the first place, the amount of BOD in the fermented liquor has been drastically reduced so that it can be discarded with less danger of overloading the streams. Secondly the SO_2 can be recovered in the stripping operation and used to prepare fresh cooking acid. Finally the desugared lignosulphonates in the fermented liquor find better markets than the whole liquor solids since in some cases the sugars may interfere with a desired adsorption of the lignosulfonates. More recently Helena Karczewska [16,17] studied the alcohol and fodder yeast production from sulphite waste liquors of various densities. The waste liquors were enriched with nitrogen and phosphorus and subjected to alcoholic fermentation for 48 hours by strains of Saccharomyces Cerevisiae Ma, Schizosaccharomyces Pombe no. 2, and Candida Tropicalis P6. The alcohol yield varied with the strain of yeast and the concentration of dissolved solids in the waste. The highest production of alcohol from purified waste liquor was achieved with the culture Schizosaccharomyces Pombe, strain Ma, which produced 68.2 ml alcohol from 100 g. of fermenting sugar. The yeast yield during the fermentation of both normal and concentrated wastes was also examined. The strains P-6 (C-tropicalis) and K-2 (C-Robusta) were used. The fermentations were carried out at 34-38°C and at a pH between 5.5 and 6.8 with continuous aeration. She found that for optimum yeast yield, the ratio of total reducing substances to the content of the solids in the

liquor should not fall below 10. Our present work which is only a preliminary part of a more general research program differs from the previously mentioned studies in that we extract the sugars from the spent sulphite liquor before fermentation.

THEORY

1. Kinetics

1.A Kinetics of yeast growth

A schematic representation of a yeast growth curve was obtained from the text of Pelczar and Reid [18] and presented in Fig. 2. The activity of the cells during each of the fermentation phases can be described as follows:

- The lag phase:
  During this phase, the population remains temporarily unchanged. The cells are active in the sense that they use substrate to synthesize new protoplasm. The cells in their new environment are deficient in particular enzymes, which are first produced in amounts required for optimal activity in the given medium. The organisms are metabolizing but there is a lag in cell reproduction.

- The logarithmic or exponential phase:
  During this period, the cells which are now used to their environment are using substrate and nutrients for reproduction. The cells are dividing at a constant rate.

- The stationary phase:
  There are several reasons why a population enters this phase. This trend toward cessation of growth usually occurs after exhaustion of some of the nutrients. Production of toxic products during the growth can also be responsible for this behaviour. The population remains constant for a time, as a result of the balancing of reproduction rate by an equivalent death rate.

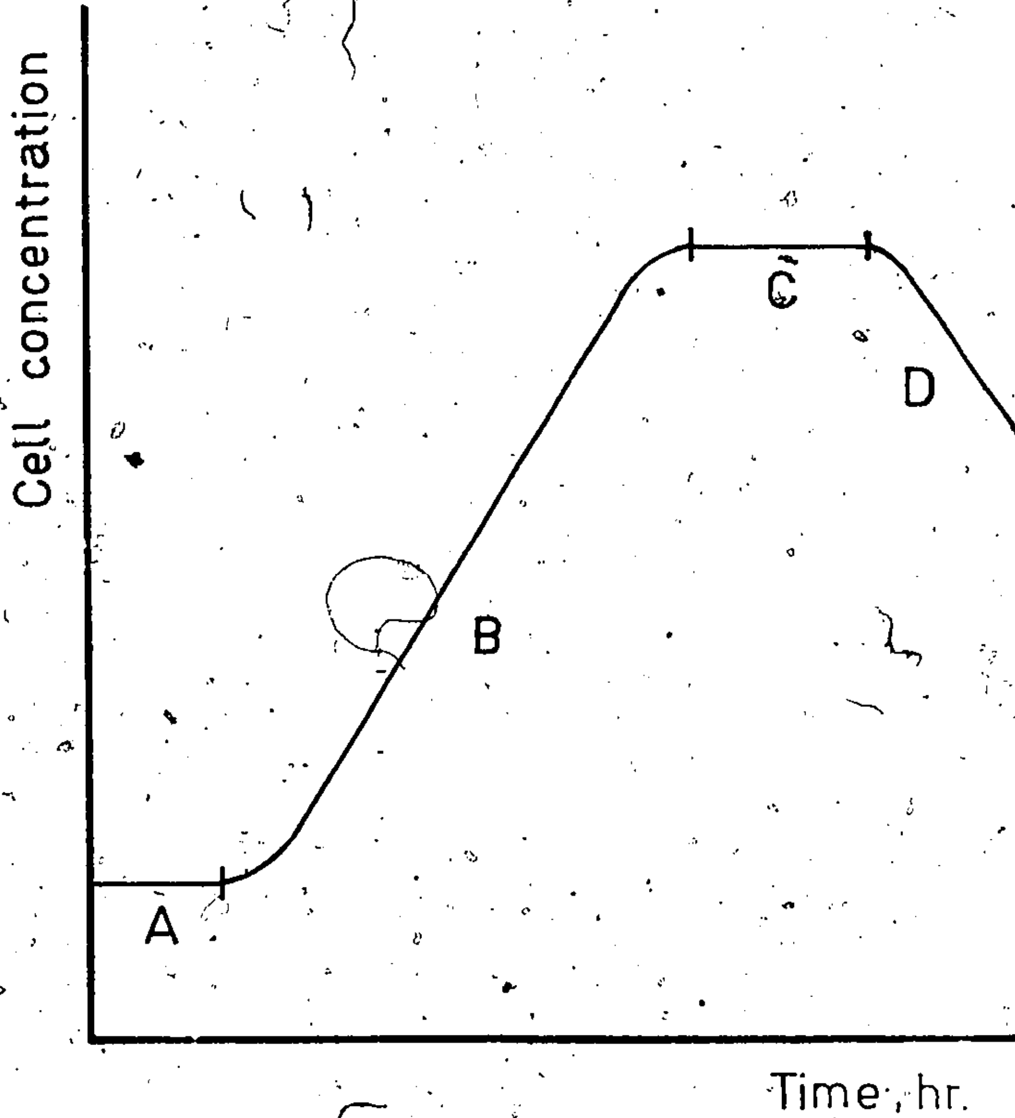

Figure 2 Typical yeast growth curve
(A) lag phase, (B) logarithmic phase,
(C) stationary phase, (D) decline phase.

The phase of decline or death:

After the just mentioned stationary period, the microorganisms die faster than new cells are produced. Accumulation of inhibitory products such as acids usualy helps promoting this situation.

Note that the culture proceeds gradually from one phase to the next. This means that not all the cells are in exactly the identical physiological condition toward the end of a given phase of growth. Obtained experimental data are plotted and discussed in a later section.

In 1972 Anderson[19] published a work on the kinetics of yeast growth. He allowed an initial charge of yeast to multiply and grow in a nutrient solution. The yeast cell volume and sugar concentrations were measured over the period of the experiments and the results compared with predictions resulting from the knowledge that after an initiation period in which the yeast becomes accustomed to a new environment, the growth rate is first order in yeast concentration and also depends on the concentrations of sugar, oxygen, available nitrogen, minerals, vitamins, hydrogen ion concentration and temperature. Hinshelwood[20] formulated the rate equation as a product of terms for each of the vital substances.

$$\frac{dY}{dt} = kY\left[\frac{b_S C_S}{1+b_S C_S}\right]\left[\frac{b_o C_o}{1+b_o C_o}\right]\cdots\left[\frac{b_x C_x}{1+b_x C_x}\right] \quad (1)$$

where Y is the yeast cell volume per unit volume of solution, k is a rate constant, C_S is the sugar concentration b_S is a constant for sugar, and so forth for oxygen (o) and others. They kept variables such as temperature and pH constant. For an excess of any of the nutrients the

product bC becomes large compared to unity and the term in brackets approaches unity. In the experiment considered, all nutrients except sugar were supplied in large excess so that the rate expression becomes

$$\frac{dY}{dt} = k\,Y \cdot \frac{b_s C_s}{1 + b_s C_s} \tag{2}$$

Since yeast growth occurs both by the growth of individual cells and by cell division with further growth and division of new cells, a rate equation for the number of cells per unit volume may differ slightly from that for the volume of yeast per unit volume. A material balance for the sugar may be used to relate sugar and yeast concentrations:

$$R\,(Y - Y^{\circ}) = C_S^{\circ} - C_S \tag{3}$$

where 1/R is the yeast cell volume which results from the utilization of a unit amount of sugar and the superscript ° indicates an initial value. Combining with equation (2) to eliminate C_S yields

$$\frac{dY}{dt} = kY \left[\frac{b_S\,(C_S^{\circ} - R\,(Y - Y^{\circ}))}{1 + b_S\,(C_S^{\circ} - R\,(Y - Y^{\circ}))} \right] \tag{4}$$

which may be integrated for the initial condition $Y = Y^{\circ}$ at $t = 0$, to give

$$Y = Y^{\circ} e^{kt} \left[\frac{Y^{\circ}}{Y} \left(1 - \frac{R}{C_S^{\circ}} (Y - Y^{\circ})\right) \right]^{\frac{1}{b_S (C_S^{\circ} + RY^{\circ})}} \tag{5}$$

As Anderson [19] noted, the growth curve of equation (5) will not include the induction period and transition at the start of growth nor the loss of cell volume after the sugar has been consumed.

1.B Kono - Asai model:

Kono and Asai [21] developed a model which classified biochemical reactions according to their production rate. The two basic equations in the model were

$$\frac{dY}{dt} = k_y \phi Y \tag{6}$$

$$\frac{d\,C_P}{dt} = kp_1 \phi Y + kp_2 (1 - \phi) Y \tag{7}$$

Y anc C_P represent cell concentration and product concentration respectively. kp_1 and kp_2 are production rate constants and ky is the growth rate constant. ϕ represents an apparent coefficient of growth activity and its value depends on the phase of fermentation. This model requires the data to be fitted to four regions, corresponding to the phases of induction, transient, exponential and declining growth. Plotting $\frac{dY}{dt}$ versus Y should then result in a triangle - like graph. For similar reasons a plot of $\frac{d\,C_P}{dt}$ versus Y should also result in a triangle - like graph. The values of kp_1 and kp_2 are then obtained from the constant slopes of the sides of the triangle representing equation (7). Table 3 shows the classification of fermentation processes according to the values of kp_1 and kp_2. Graphs representing our data by this model are discussed in a later section.

Table 3 Types of fermentation processes classified according to the values of production rate constants, kp_1 and kp_2, in general formula of production rate, Eqn. (7).

kp_1	kp_2	description
+	+	product formation associated with growth and nongrowth
+	0	product formation associated with growth
0	+	product formation associated with nongrowth
+	-	product formation associated with growth and decreased with nongrowth.

2. Sugar transport

Lehninger [22] suggested a possible substrate specificity of a so called "sugar carrier", which is a component of the cell membrane of the yeast, that forms a complex with a sugar molecule in order to transport it into the cell for digestion. Cirillo [23] studied the relationship between sugar structure and competition for the sugar transport system in bakers' yeast. He attempted to relate the activity of each sugar with the way its structure differs from that of D-glucose, which showed the highest activity for inhibition of L-sorbose transport. Single changes at each of the carbons of D-glucose resulted in variable decreases in activity depending upon the carbon number and the alteration. The combination of two or more changes introduced a decrease which was greater than the decrease in activity resulting from the individual changes occuring alone. This theory is described further in the discussion of results with reference to this work.

3. Factorial design

The detailed study performed on dextrose was based on basic knowledge of statistical experiment-design. An explanation of the factorial design technique which we used, was given by Pavelic and Saxena [24]. This technique was developed to increase research efficiency. The disadvantages involved in designing experiments on the basis of varying one variable at a time can be summarized as follows:

1. Many-experiments are required, which are time - consuming and expensive.
2. The interaction between variables is not determined i. e. insight into their simultaneous effect is not gained.

A logical experimental program for practical study of physical systems is factorial design. Here experimental conditions are chosen by selecting a fixed number of levels for each variable, after which experiments are run at all possible combinations. However the basic assumption of linearity can be troublesome. In a 2^k design, the 2 represents the number of levels (high and low) and k the number of variables (x_1, x_2, x_3). A coding system is used to simplify writing all the possible combinations. This system uses + 1 for the high level and -1 for the low level. Since all possible combinations for two levels of three variables require eight tests, one way to represent this is by considering our example's three variables as three mutually perpendicular coordinate axes (x_1, x_2 and x_3). The 2^3 factorial design can be represented geometrically as a cube (Fig. 3). The eight corner points of the cube represent the eight test conditions, while the origin of the system (0, 0, 0) represents physically the midvalue conditions of x_1, x_2 and x_3. **Essentially**, what we want to know is which variables

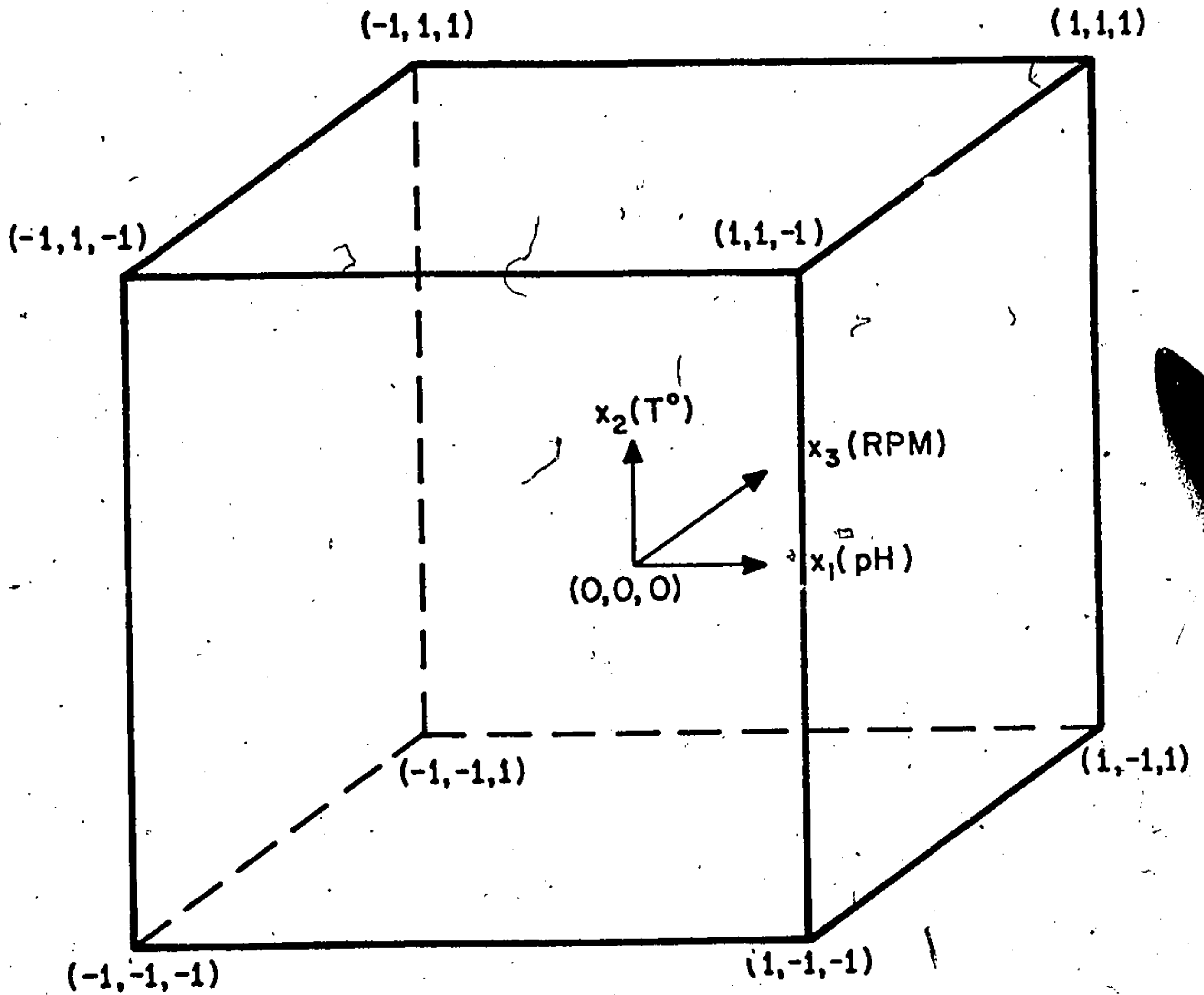

Figure 3 Factorial design 2^3 in geometric form

(or combinations thereof) are important for the process. This information may be obtained by computing average effects of each variable. Geometrically, the average effect of x_1 is simply the difference between the average result on a plane at high level of x_1 and the average result on a plane at low level. We represent this overall x_1 effect (E_1) as follows:

$$E_1 = [(\Sigma \text{ y's at high } x_1 \text{ level}) - (\Sigma \text{ y's at low } x_1 \text{ level})] /4 \qquad (8)$$

The same holds true for the average x_2 effect and the average x_3 effect. To calculate the interaction between x_1 and x_2, we can visualize the geometrical representation cube as being compressed in the direction of x_3, thereby transforming the cube into a two-dimensional square. The values at the four corners of the square are the average results of two tests. The two diagonals represent the high-and low-level conditions. The interaction between x_1 and x_2 can be calculated by taking the average results of the diagonal representing the high level conditions (A) and substracting the average results of the diagonal representing the low-level conditions (B). So

$$E_{12} = [(\Sigma \text{ y's at A}) - (\Sigma \text{ y's at B})] /4 \qquad (9)$$

Similarly for E_{13} and E_{23}. Similar techniques can be applied to compute three-factor interactions between x_1, x_2 and x_3 (E_{123}). Although the graphical - representation approach (cube method) is practical for the average effect as well as for the two - factor interactions, the usefulness of the method is somewhat limited because if it is applied to more than three variables it becomes cumbersome, if not impossible to use. We shall therefore use a simplified calculation procedure that is easily applied to the analysis of two-level factorial designs involving any number of variables. This will be demonstrated in appendix 6.

SOURCE OF CHEMICALS AND MATERIALS

The following chemicals were used:

Fisher certified D (+) glucose (dextrose), D (+) mannose and D(+) galactose. Fisher Chemicals, Ottawa.

Hydrochloric acid:	Macco reagent 6066. McArthur Chemical Co., Montréal
Ammonia	Macco reagent 6330. McArthur Chemical Co., Montréal
Sulfuric acid	Allied Chemical reagent. Allied Chemical Canada, Pointe Claire, Qué.
Phenol	Analar analytical reagent. BDH Chemicals, Poole, England.
Yeast nitrogen base (Y. N. B.):	containing all the essential nutrients and vitamins for the cultivation of yeast except a source of carbohydrate was obtained from DIFCO laboratories. Its composition can be found in appendix 1.
Yeast	commercial Fleischmann's dry yeast containing a mixture of strains of Saccharomyces Cerevisiae was used throughout this study.

APPARATUS

1. Fermentor:

The fermentor used for our experiments was a New Brunswick Scientific Co. (N.B.S.) modular microferm bench top fermentor model MF-114 with a 5 l. capacity. The diameter of the vessel was 5 $\frac{10}{16}$" its height was 12 $\frac{3}{16}$". Agitation was achieved by double impellors, each with 6 flat stirring paddles. They were situated 5" apart and had a diameter of 2". The lower impellor was situated 2 $\frac{1}{2}$" from the bottom of the vessel and $\frac{3}{4}$" above a single orifice air sparger of $\frac{3}{16}$" diameter. A four-tube baffle assembly served as a heat exchanger and was also utilized for the introduction of air. The baffles had a width of $\frac{3}{4}$" and a thickness of $\frac{1}{8}$". The unit had a built-in strip-chart temperature recorder, as well as an automatic foam controller. Details are shown in Figure 4.

2. Autoclave:

A vertical N.B.S. autoclave, model AE 15-10, was used for sterilizing the fermentor. Pressure could be adjusted up to 25 psig, and automatic control was assured by a pressure relief valve.

3. pH controller:

Continuous recording and control of pH was achieved to within 0.1 pH units automatically with an N.B.S. modular pH controller model pH-22. Two persitaltic addition pumps added acid or base on demand. Steam sterilizable pH electrodes with stainless steel immersion holders were mounted in threaded fittings in the fermentor headplate and connected to the pH instrument.

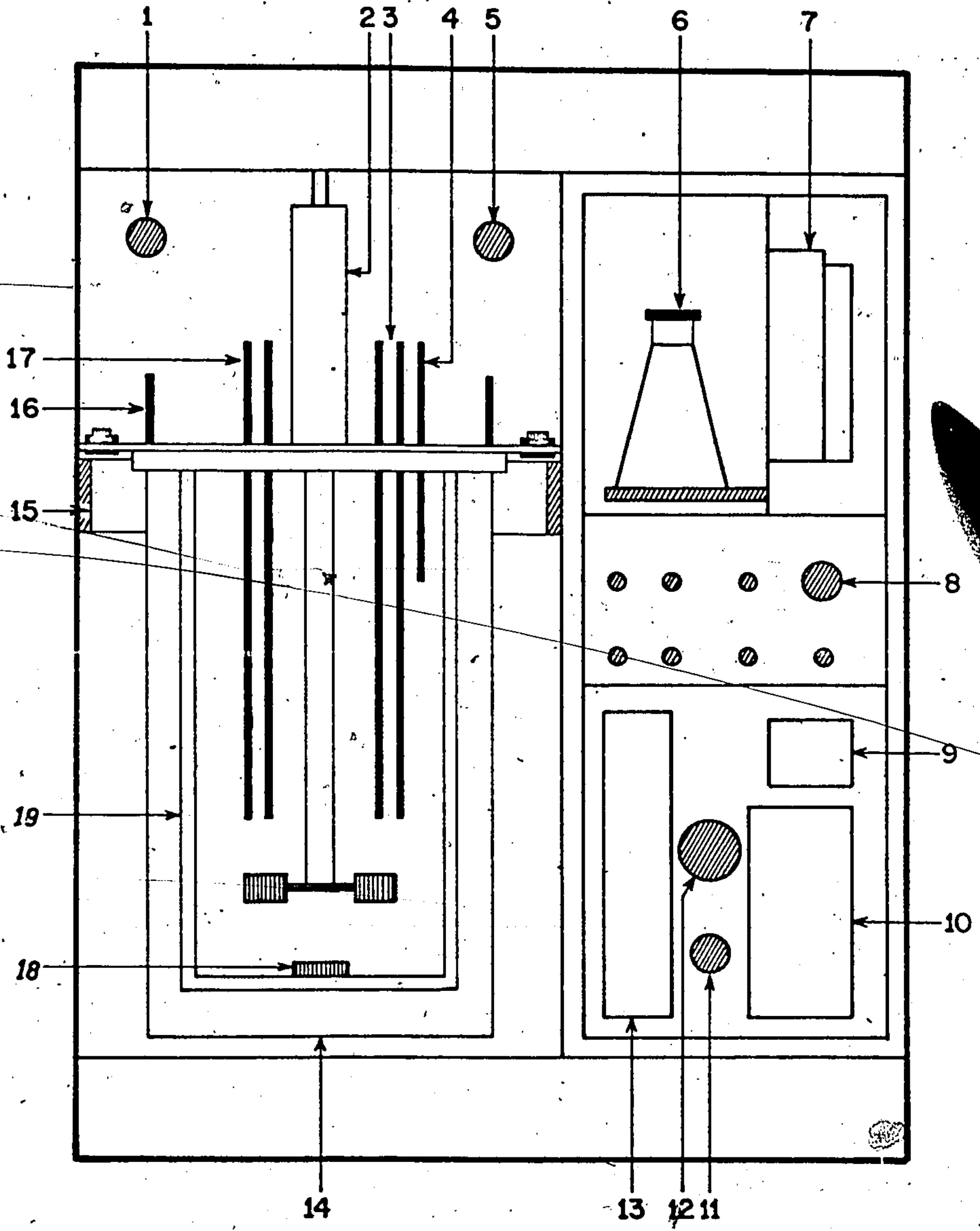

Figure 4 Fermentor component locations

<u>Key to fermentor component locations</u>

1	filter for outgoing air
2	variable speed drive and impellor shaft. Only one impellor is shown.
3	pH electrodes
4	antifoam line
5	filter for incoming air
6	antifoam reservoir
7	antifoam pump
8	temperature control
9	tachometer
10	temperature recorder
11	air pressure regulator
12	air pressure gauge
13	flow meter
14	5 liter vessel
15	vessel support plate
16	water line
17	thermocouple
18	single orifice sparger
19	baffles

4. Oxygen analyzer:

An N. B. S. model D. O. - 50 analyzer was used together with an N. B. S. steam-sterilizable electrochemical membrane-type oxygen electrode model M1016-0201. Its performance is explained in the discussion of the experimental results.

5. Inoculator - sampler:

An N. B. S. inoculator-sampler, model S20, was used. It consisted of a removable glass collecting tube and a built-in air filter chamber. Inlet and outlet lines, connected to a sterilizable plastic syringe pump, were used for pumping liquid into and out of the fermentor.

6. Centrifuge:

A model HN-S table top centrifuge from International Equipment Co. was used in the determination of yeast concentrations, as described in the experimental procedure.

7. Gas chromatograph:

Ethanol concentrations were determined using a model 90-P3 Varian Aerograph gas chromatograph with a thermal conductivity detector. The 10' long, 0.25 inch column was obtained from Chromatographic Specialties, Brockville, Ontario, and was packed with Chromosorb-W (30-60 mesh) coated with Carbowax 20 M. For chromatograph settings; see the section on experimental procedure.

8. Spectrophotometer:

The sugar concentration was followed using a Bausch and Lomb precision spectrophotometer n°. 33-26-50. Quartz cuvettes with a 1 cm light path were used. The analytical method is described in the experimental procedure.

EXPERIMENTAL PROCEDURE

1. Startup of fermentor:

The growth medium employed consisted of 100 g of sugar and 40 g of yeast nitrogen base dissolved in distilled water. The fermentor vessel with this dissolved growth medium, the air inlet, the exhaust filters, the transfer tubing, the antifoam reservoir and the pH-electrodes was sterilized in an preheated autoclave for 5 minutes at 90°C. The total residence time in the autoclave was about 30 minutes. The sterilization conditions described above were reported by Anderson [19] to be sufficient, due to the resistance of yeast to disease, its rapid growth rate which discourages contaminants and the acidic character of the medium. Following the sterilization the fermentor was replaced in the microferm. The impellor shaft was coupled to the drive, and the pH electrodes and the filters were connected. The impellor speed, the air flow and the growth media temperature and pH were adjusted to the required level and the yeast inoculum was then introduced. This inoculum consisted of 4 g of yeast suspended in sufficient distilled, sterile water which, when added to the vessel, made a 3 liter solution. Automatic pH control was achieved by adding 2 N HCl or 4 N ammonia as required. These concentrations were chosen because their volume compensated for the removed sample volume so that no correction for dilution was needed.

2. Sampling:

Samples were taken at intervals of 1 to $1\frac{1}{2}$ hours. The fermentor was equipped with a stainless steel sampling tube terminating near the bottom of the jar. The sampling device was connected to this

tube. Representative samples were pumped from the fermentor with a plastic syringe to a collecting tube which was fitted with a bacterial filter. Representative samples were assured by recirculating three tube volumes (about 10 ml) before taking a sample, eliminating the effect of dead volume in the sample tube.

3. Yeast analysis:

Ten millilitre samples were centrifuged for 5 minutes at 3000 rpm to produce a compact yeast mass at the bottom of the centrifuge tubes. Concentrations were reported in volume percent following Anderson's [19] procedure. The supernatant liquid was collected for subsequent sugar and ethanol analysis. A drop of phenol was added to prevent the remaining yeast from changing the sugar concentration and the solution was frozen until the sugar analysis could be performed.

4. Sugar analysis:

The sugar determination was made by the colorimetric method of Dubois et al. [25]. The samples were diluted 1000 times, one ml of a 5% phenol solution was added to 2 ml of diluted sample, and this was further mixed with 5 ml of concentrated H_2SO_4. This solution was then cooled to room temperature and the transmittance of the samples and of a standard solution were measured at 490 nm on the spectrophotometer described earlier.

5. Analysis for alcohol:

The alcohol analysis was performed by means of a gas chromatograph with a thermal conductivity detector, using specifications published by Aiyar and Luedeking [26]. The column, described earlier,

was maintained at 180°C, the detector at 240°C and the injector at 200°C. The filament current in the detector was kept at 150 mA. Helium at 20 psi was used as the carrier gas. The sample volume was 1 µl for all determinations. Calibration curves for the sugar and ethanol analysis shown in Figures 5, 6, 7 and 8 can be found in Appendix 2.

EXPERIMENTAL RESULTS AND DISCUSSION

This section deals with significant and illustrative results obtained in the course of this research.

It is written in three parts, corresponding to:

1. Fermentations of glucose based on the experiment published by Anderson [19].

2. Fermentations of glucose, designed to yield optimal conditions for ethanol production.

3. Fermentations of glucose, galactose and mannose using the conditions obtained from part 2.

1. Results of part 1

1.A Measurements:

The first part of the experimental work consisted of fermenting glucose under the following conditions:

- temperature = 29°C
- pH = 5.0
- rpm = 400
- air flow Q = 7000 cc/min

in the fermentor described earlier.

The chemicals consisted of:

- 40 g yeast nitrogen base
- 100 g glucose
- 4 g yeast (Saccharomyces Cerevisiae)
- distilled water to make 3 l solution.

The purpose of these runs, which were based on the experiment published by Anderson [19], was to test the newly assembled equipment and to become familiar with the techniques involved in order to obtain

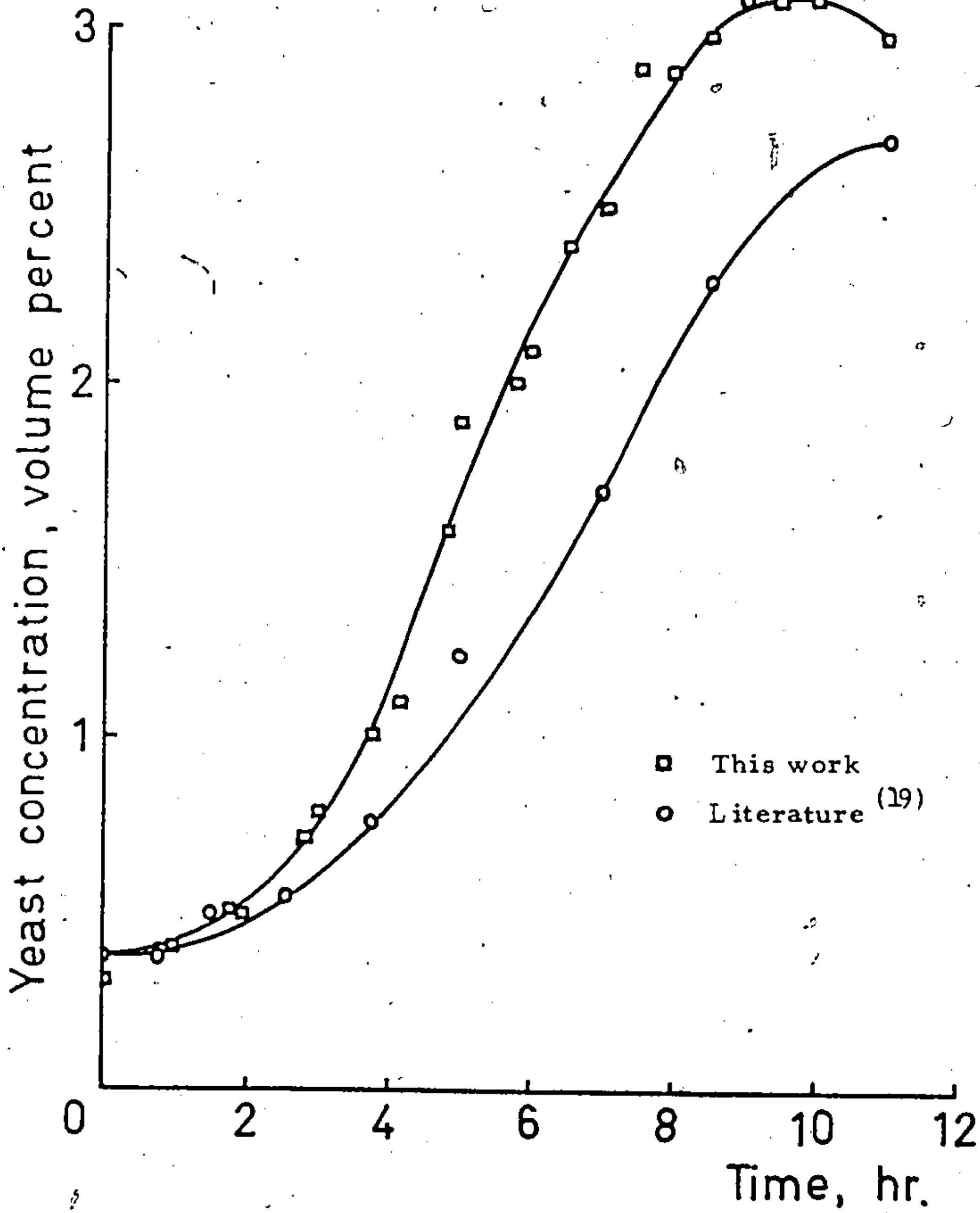

Figure 9 Growth of Yeast

reproducible results. The numerical results of this first part can be found in appendix 3 .

1. B Discussion

1. B. 1. Yeast growth:

Figure 9 compares our data on yeast growth with those obtained by Anderson [19]. Our curve was obtained by fitting the measured data, of three runs, for yeast concentration, presented in Appendix 3 , to a third order equation by a program for least squares polynomial regression. Figure 9 shows that we produced more yeast; the following reasons can be quoted to explain this:

a) In his paper, Anderson only mentioned the highest possible reading on the rotameter included in his air feed line. Our experiments were conducted at this maximum value for air flow (7000 cc/min) but we cannot be certain of the value for the flow rate of Anderson's experiments, and since yeast production is an aerobic process, a difference in air flow could be partly responsible for a difference in yeast production.

b) Anderson did not specify any mixing data except for his stirrer rpm, which was much lower than ours. If all other factors were equal, our mixing and thus oxygen transfer was superior.

1. B. 2. Sugar Analysis

Anderson reported that he used a modification of Nelson's [27] colorimetric method for determining the sugar concentrations. In an attempt to modify this lengthy method, designed for the determination of glucose concentrations in blood,

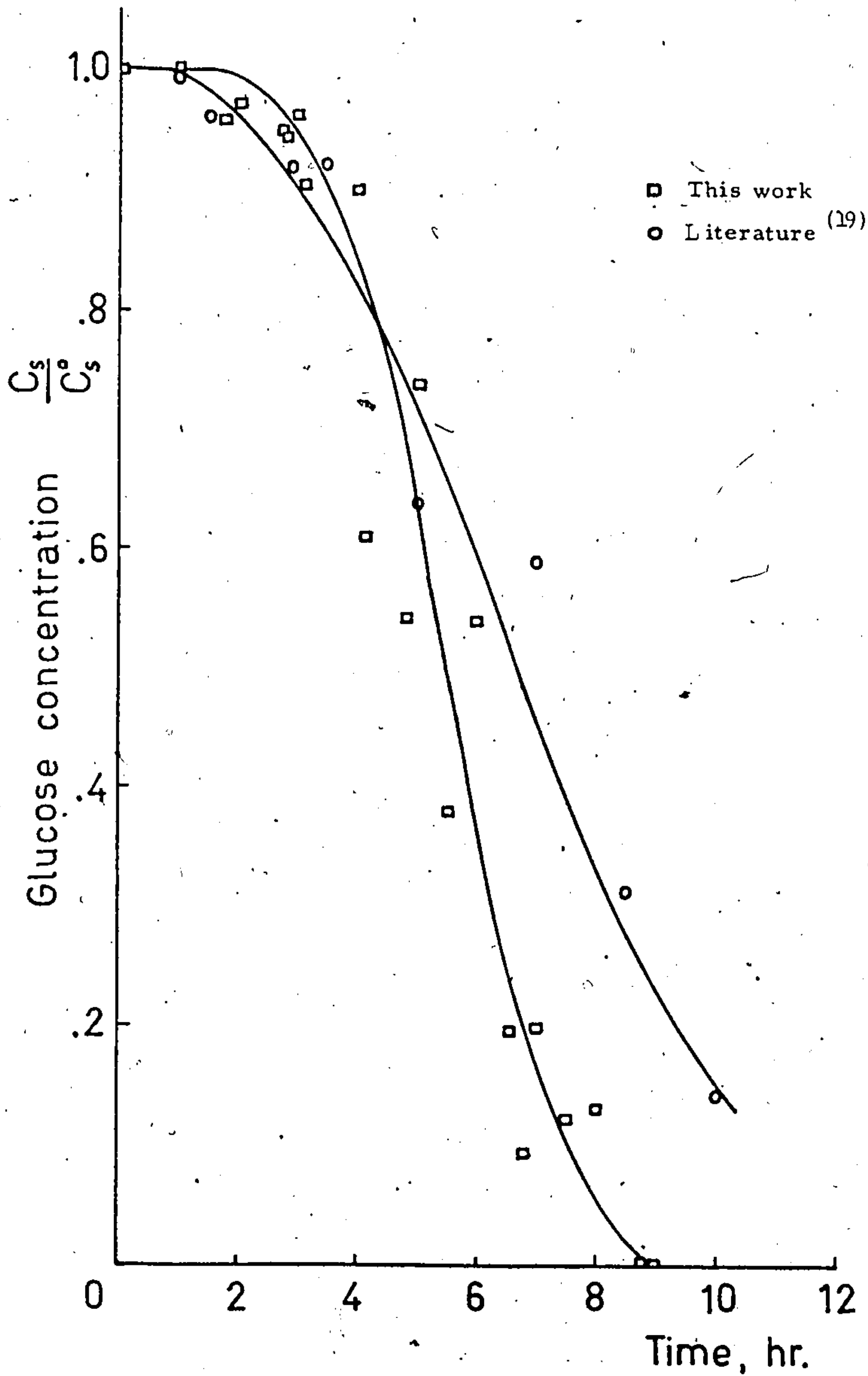

Figure 10 Consumption of Sugar by Yeast

the filtration step was omitted but this modification and others did not result in a linear calibration curve of transmittance versus sugar concentration. Meanwhile the less time - consuming method of Dubois et al.[25] described earlier was found which could be used for all hexoses, and which was used for all sugar concentrations in this study. Figure 10 compares the results of this study with those obtained by Anderson. The curves were normalized because the initial sugar concentration reported in Anderson's paper was different from the one shown in his graph. Our curve was obtained using the same procedure as described for the yeast curve. The difference between the two curves is in agreement with the findings from the yeast curves; the higher yeast production which was obtained resulted in a more rapid depletion of substrate concentration.

1.B.3 Oxygen analysis:

A probe was used to determine the dissolved oxygen concentration during the course of the experiments, because R. Finn[28] has suggested that perhaps oxygen availability could limit the growth of yeast which implies that equation (2) would not be applicable since it implies an excess of all necessary constituents except sugar. Reproducible data could not be obtained on oxygen concentration and a great deal of work was put into designing a reliable calibration method which would allow reproducible results. Two probes were used during the course of this work in order to achieve this goal since the first probe which was supposed to withstand 60 sterilizations did not survive 20 heat-treatments. A satisfactory calibration method for the D.O. probes was finally obtained, which in more recent runs proved

to yield reproducible results. This method consisted of setting the span (0-100% dissolved oxygen) in the following way: after the sterilization, the medium was aerated and mixed for a few minutes until a constant value for dissolved oxygen was recorded; this value was set to 100%. The next step consisted of zeroing the instrument, assuming that the mechanical zero was equivalent to zero dissolved oxygen. This method of zeroing was checked against the zero obtained by introducing an excess sodium sulphite (a desoxygenation product). The error made was less than 3%.

2. Results of part 2

2.A Measurements:

The second part of the experimental work was devoted to experiments with glucose. The effect on ethanol yield of three variables; pH (x_1), temperature (x_2) and rpm (x_3) was studied using factorial design. We therefore performed 2^3 runs, which were duplicated so as to add up to 16 experiments. As in the first part, the same amount of the same chemicals was fermented per run. The maximum rate of yeast production was found, from the results of part 1, to occur after about 4.50 hr. We therefore bubbled air through to build up a large yeast population which then could produce ethanol anaerobically. As in the first part, samples were taken to determine the concentrations of yeast, sugar and ethanol. Plotting the collected data resulted in a graph as shown in Figure 11, which is a typical plot for glucose fermentations. This graph is not convincing regarding the existence of a lag period in the yeast growth. The first point for yeast concentration, reported after 0.25 hr was 0.3 vol.%. This value could be misleading, since it is very difficult to measure low yeast concentrations accurately in 10-ml centrifuge tubes. After 4.50 hours the air flow was stopped and the reaction proceeded

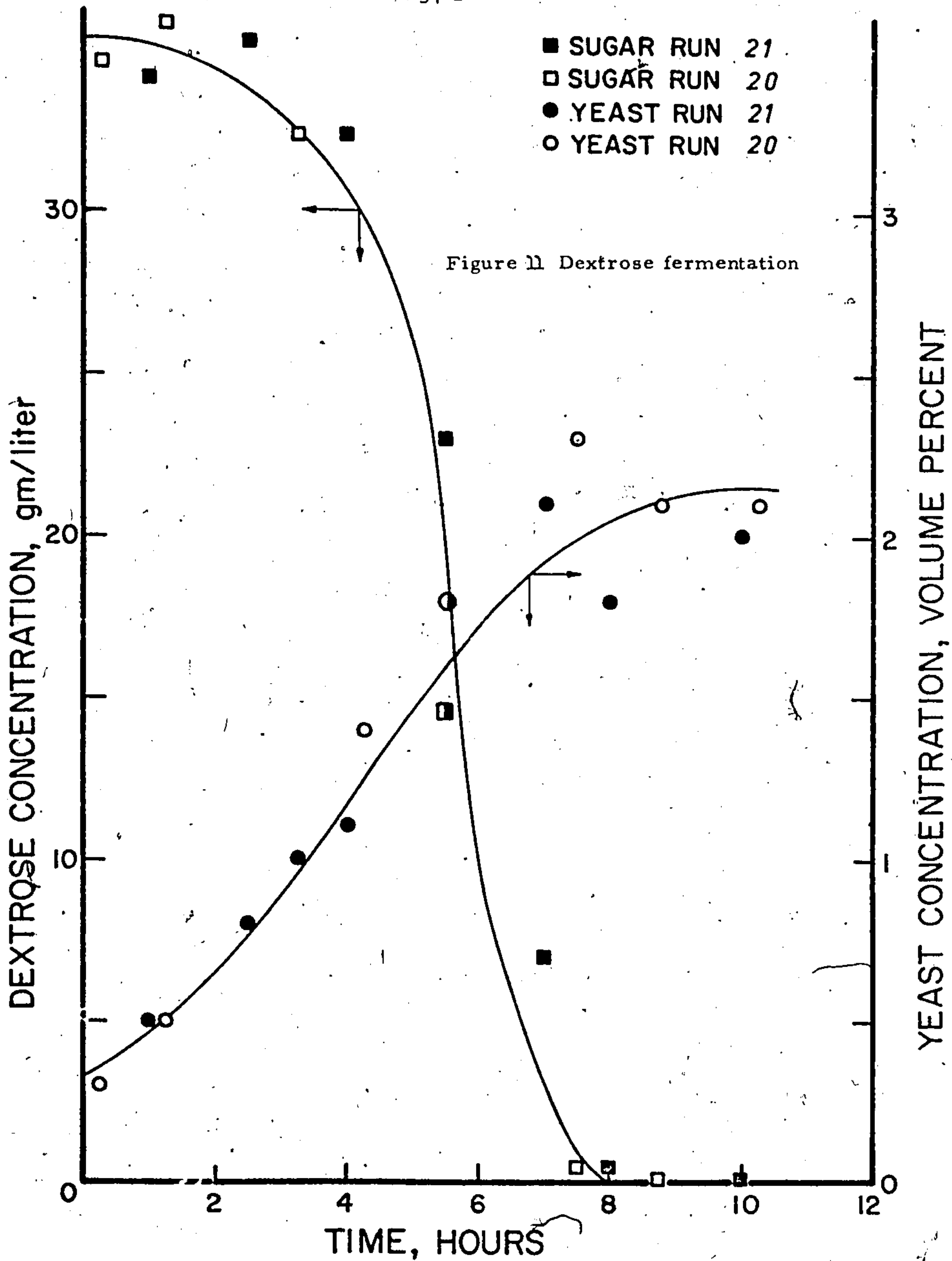

Figure 11 Dextrose fermentation

anaerobically to produce ethanol. In Fig. 12 it can also be seen, that in the case of a glucose fermentation the maximum rate of yeast production was obtained after about 4.50 hr. Since an increasing amount of yeast cells were present in the medium, the sugar concentration decreased with time to reach a maximum rate after about 5.50 hr. After eight hours of fermentation, all the sugar was consumed. It can also be seen that at this time the yeast curve started to proceed horizontally which as mentioned in the theoretical section is an indication that the rate of birth of new cells equals the rate of death. In an effort to determine the optimal conditions for ethanol production calculations were based on the ethanol concentration at zero sugar concentration since it was desired to obtain this condition from a pollution control viewpoint. The results of this second part can be found in appendix 4. The values for all runs of the ethanol concentrations at zero sugar concentration are represented in Table 4.

TABLE 4 : Results of ethanol concentration at zero sugar concentration in ml/l for the runs of part 2

	pH : 4		pH: 6	
	rpm: 500	rpm: 700	rpm: 500	rpm: 700
T° = 27°C	13.6	12.2	12.7	16.0
	14.5	13.0	13.2	14.1
T° = 32°C	14.8	16.4	15.2	16.7
	15.8	14.5	13.5	15.6

For test no. 1, with the following conditions: T° = 27°C, pH: 4, and rpm: 500, the average ethanol concentration at zero sugar concentration is

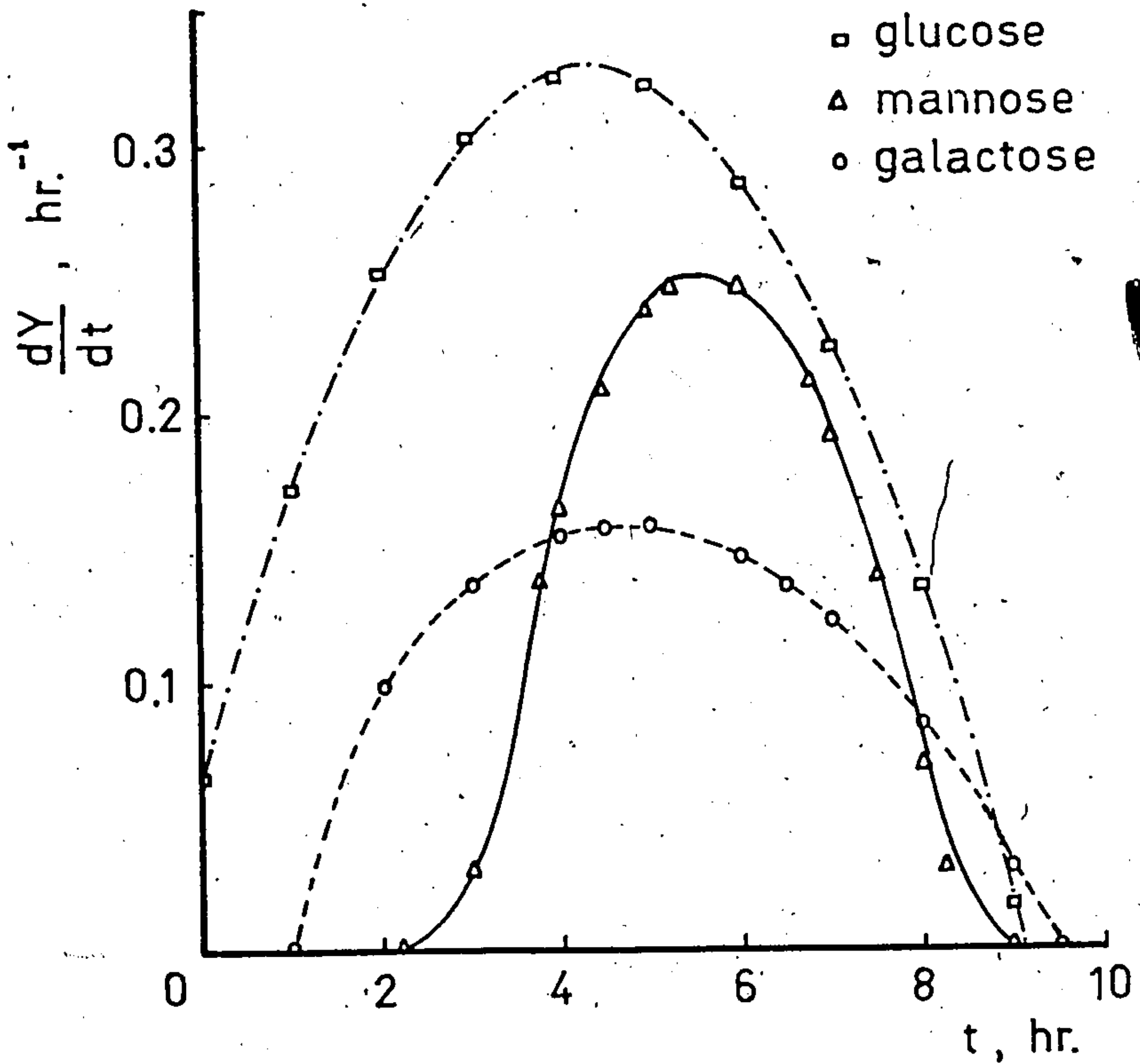

Figure 12 Rate curves for yeast growth

$(13.6 + 14.5)/2 = 14$ ml/l. The corresponding values for the other tests are given in Table 5.

TABLE 5. Average ethanol concentration at zero sugar concentration

Test No.	rpm	pH	T°	Alcohol concentration
1	500	4	27	14
2	500	6	27	12.9
3	500	4	32	15.3
4	500	6	32	14.3
5	700	4	27	12.6
6	700	6	27	15
7	700	4	32	15.4
8	700	6	32	16.1

Figure 13 shows the planes of low and high pH level from which E_{pH} was calculated. The test numbers as well as the average ethanol concentrations are also indicated. For example, the average pH effect E_{pH} can be calculated as:

$$E_1 = E_{pH} = \frac{(12.9 + 14.3 + 15 + 16.1) - (14 + 15.3 + 12.6 + 15.4)}{4} \qquad (10)$$

or $E_{pH} = 0.25$

However, a more convenient method based on vector multiplications was used to calculate the effects of the variables and of their interactions. Examples of these calculations can be found in appendix 6. They yielded the following results:

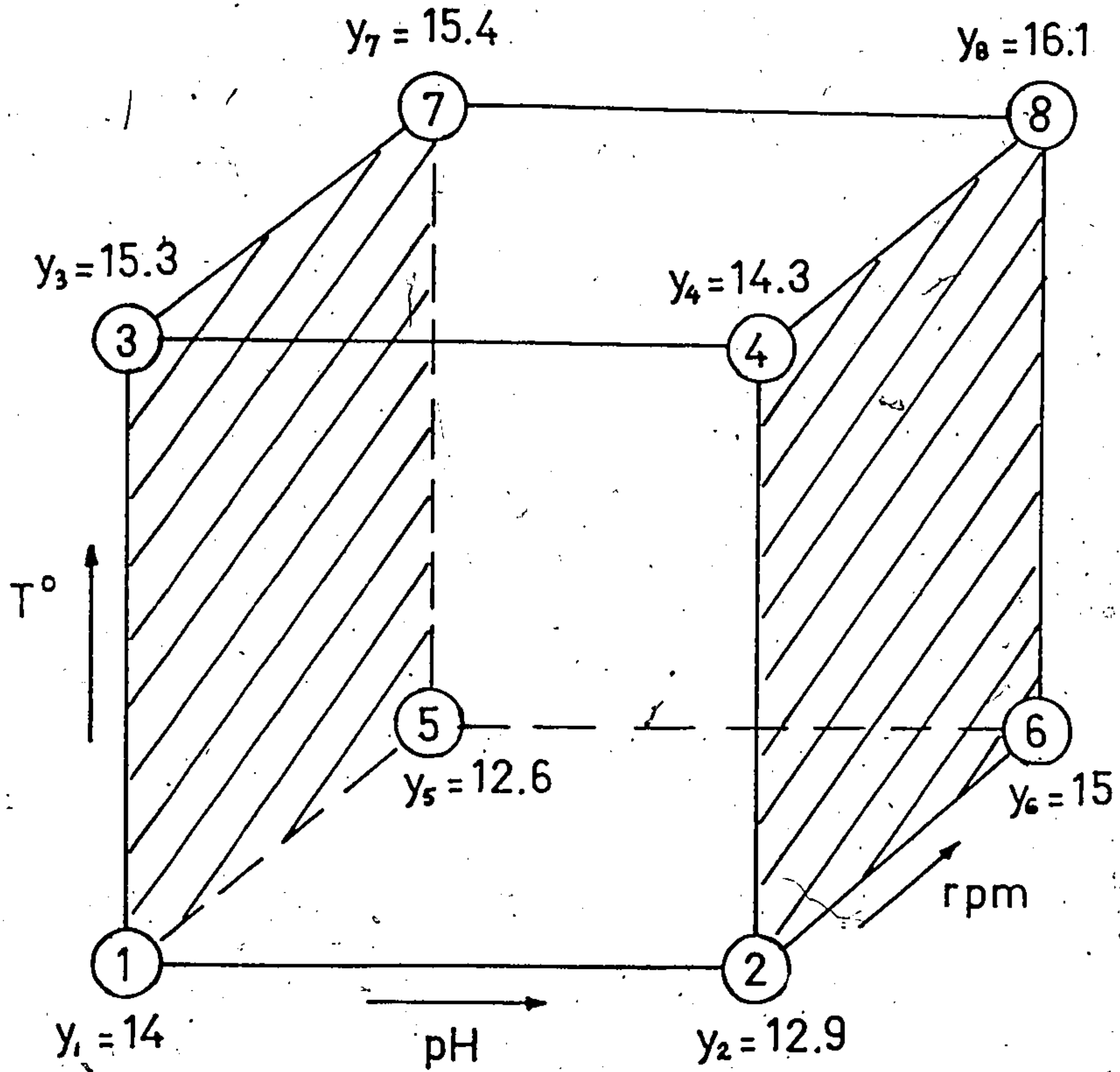

Figure 13 Average pH effect

- average pH effect = $E_1 = E_{pH} = 0.25$
- average T° effect = $E_2 = E_{T°} = 1.65$
- average rpm effect = $E_3 = E_{rpm} = 0.65$

For the two-factor interactions we obtained

$E_{pH, T°} = -0.40$
$E_{pH, rpm} = 1.30$
$E_{T°, rpm} = 0.30$

and for $E_{pH, T°, rpm}$ a value of -0.45 was found. The average ethanol concentration was found to be 14.45 with a standard deviation σ of 1.23.

2.B Discussion:

2.B.1. Factorial Design:

The statistical analysis showed that by ranking the variables according to their numerical values, temperature is the most important. Let us consider the meaning of these values

a. Average effects:

In performing the tests we measured the effect of three variables on yield of ethanol in y units. For example test no. 6 of Figure 13 with conditions rpm:700, pH: 6 and T°: 27°C produced 15 units of response. By computing average effects of each variable we determined how the individual variables rank with respect to their influence on the process. For instance, one way to proceed to evaluate the influence of pH on yield is to observe in Figure 13 that for tests 1 and 2 the conditions of rpm and temperature are the same, but the pH conditions are different. Therefore, the difference in the results

of these two tests can be attributed solely to the effect of pH. Similarly, the test conditions for test pairs 3-4, 5-6 and 7-8 are similar with respect to rpm and temperature, but different with respect to pH. Thus the differences in the results within each of these pairs reflect the effect of pH alone. We can average these four differences to calculate the overall average pH - effect E_{pH} as given by Equation 10. Similarly for the average temperature - effect E_{T° and the average rpm-effect E_{rpm}.

b. Two-factor interactions:

Let us consider the variables pH and temperature. If the effect of changing pH is the same for both levels of temperature, there is no two-factor interaction between pH and temperature. As pH and temperature act independently of each other. On the other hand, if the effect of changing pH is not the same for both levels of temperature there is a two-factor interaction between pH and temperature. In this case the effect of one variable, depends on the level of the other one. Since none of our two-factor interactions was equal to zero, we knew that our three variables did interact.

c. Test results:

Since the values of E_{pH}, E_{T° and E_{rpm} suggested that a higher yield could be expected at the high level of the individual variables, we expected test 8 of Table 5 to give the highest yield, which it did. However, the negative value of $E_{1,2,3}$ suggested a non-linearity. In the literature we found that this effect could be attributed to the pH since Aiyar

and Luedeking [26] who studied this reaction at seven different pH levels reported a maximum ethanol production at pH 5.0. This also explained the negative value obtained for the interaction of pH and temperature. We therefore designed the final experiments of part 3 so as to obey the conditions of test 8 of Table 5 except for the pH level which was set at 5.0 instead of 6.0. The ethanol result obtained for the glucose fermentations was 17.9 ml/l. This result is indeed better than the 16.1 ml/l obtained from test 8.

2.B.2 Ethanol measurements:

As previously mentioned the ethanol concentration was determined by gas chromatography, through thermal conductivity detection. This procedure resulted in recording an ethanol-peak followed by a water-peak. Since the ethanol concentration was low, the water-peak occupied a tremendous area and it took more than one hour after each injection before the pen returned to the baseline. Two injections per sample were needed in order to report average concentrations. We therefore only processed a few samples per run.

2.B.3 Yeast growth:

All the runs in this research were done using the same yeast. This yeast aged in storage so that in later runs older yeast was used. Near the end of the first runs, the yeast concentration was about 3%. The yeast concentration near the end of later runs was found to drop until it reached a constant value of about 2%. For several months the yeast concentration near the end of the runs was about 2%. This phenomena does not affect our results for ethanol production. The final ethanol

concentration is not directly related to the yeast production. The enzymes present in the medium produce ethanol under the anaerobic conditions in the second part of the fermentation until all sugar is exhausted. From the results of the identical runs 4 and 17, which were performed with a time difference of more than three months, we can indeed observe that the ethanol production is reproducible although the maximum values of yeast concentration differ considerably due to the aging effect.

3. Results of part 3

3.A Measurements

The third part consisted of six fermentations; two with glucose, two with mannose and two with galactose. As explained in the discussion on factorial design of part 2 we chose the following conditions for these runs : T° = 32°C, rpm = 700 and pH = 5.0. The results of these runs are presented graphically in Figures 11, 14 and 15 and numerically in Appendix 5. The average ethanol concentration for the glucose fermentation was 17.9 ml/l. In the case of galactose and mannose, the sugar concentration did not reach zero and we have therefore reported the average ethanol concentrations and remaining sugar concentrations after 9 hours. The mannose fermentation yielded an ethanol concentration of 7.5 ml/l with a remaining sugar concentration of 17.2 g/l. The galactose fermentation yielded an ethanol concentration of 6.5 ml/l with a remaining sugar concentration of 14.2 g/l. As before, the initial sugar concentration was 33.3 g/l. The ethanol concentration at zero sugar concentration for mannose and galactose was roughly estimated from the available data by plotting $\frac{C^\circ_S - C_S}{C^\circ_S} \times 100$ versus $\frac{C_p \times f_1}{C^\circ_S} \times 100$, which gave the concentration of alcohol as a function of the

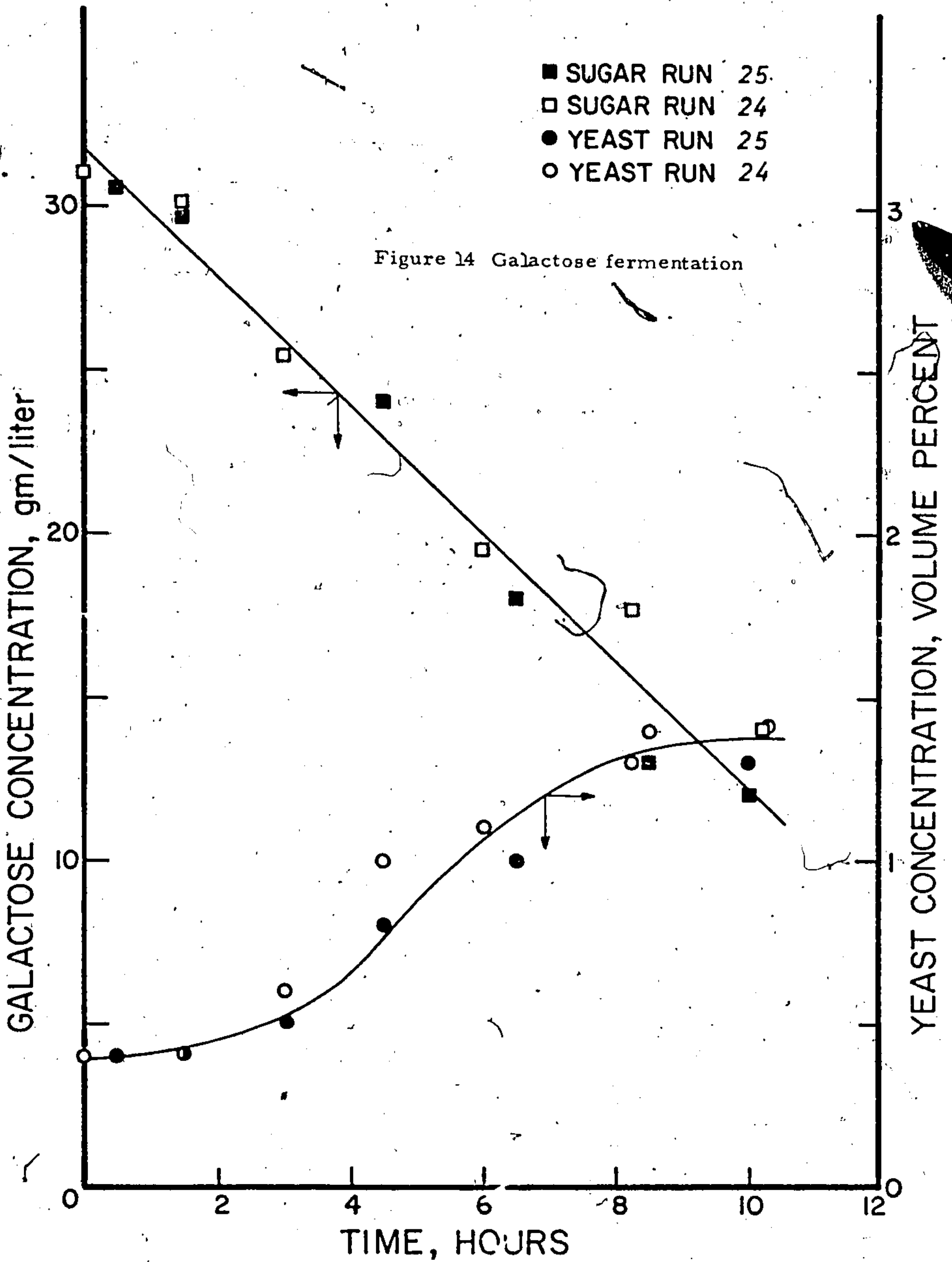

Figure 14 Galactose fermentation

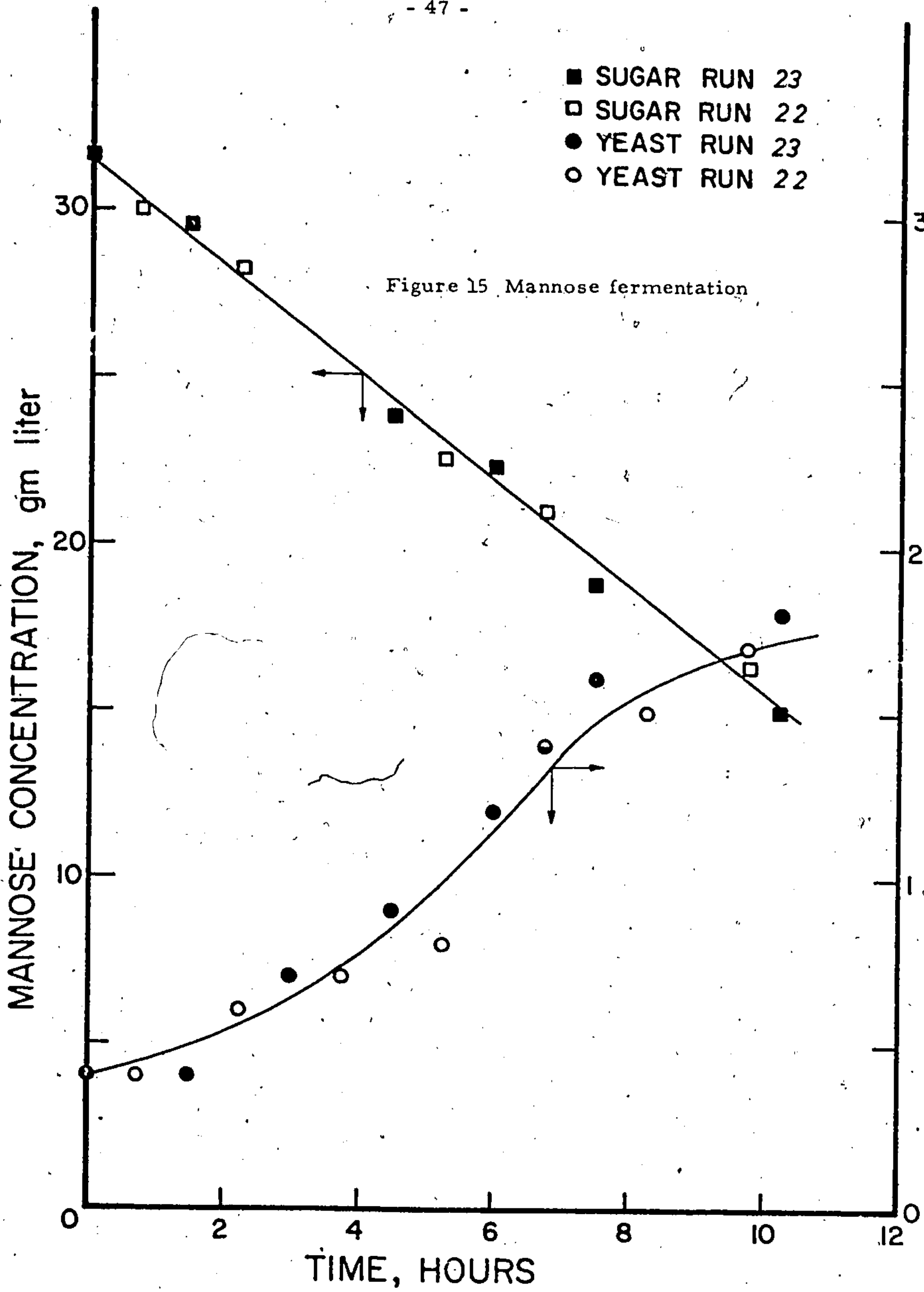

Figure 15 Mannose fermentation

percentage sugar converted. The factor f_1 (sugar to alcohol) was calculated from the stoichiometry of the reaction to be 1.565 gm sugar/ml alcohol. For the galactose fermentation, an ethanol concentration of about 10 ml/l was found by extrapolating for zero sugar concentration. Similarly in the case of the mannose fermentation, the ethanol concentration was found to be about 15 ml/l.

3. B Discussion

3. B. 1. Sugar transport:

The fermentations of galactose and mannose differed from the glucose fermentations in that, as can be observed in Figures 14 and 15, the curves representing the change of sugar concentration as a function of time were linear and did not reach the zero sugar concentration point during the entire fermentation period. We therefore reported results for ethanol production after 9 hours of fermentation. This particular behaviour could be explained by a marked substrate specificity of the sugar carrier. The work of Cirillo, mentioned previously, revealed that both D-mannose, which differs from D-glucose because it has an axial 2 - OH group instead of an equatorial 2 - OH group in its pyranose chair conformation, and D-galactose which differs from D-glucose because it has an axial 4 - OH group instead of an equatorial 4 - OH group, had decreases in activity of transportation into the cell of approximately the same magnitude. In this study, a higher rate of yeast growth (Fig. 12) and a higher rate of alcohol production was observed for glucose over mannose and galactose, in confirmation of his hypothesis.

3. B. 2 Kinetics:

With the results of Figures 11, 14 and 15 we also tested kinetic models to describe the reactions.

a. Michaelis - Menten model:

As described by many authors such as Aiba et al.[29], Webb[30], and Conn and Stumpf[31], classical graphs such as the Monod[32] graph, of velocity of reaction versus substrate concentration and the Lineweaver - Burk graph can be plotted in order to determine the values for V; the maximum reaction rate, and K_m; the Michaelis-Menten constant. These plots illustrate the Michaelis-Menten model represented by equation (11)

$$v = \frac{V C_S}{K_m + C_S} \qquad (11)$$

where v = rate of product formation

V = maximum rate of production

K_m = Michaelis-Menten constant

C_S = substrate concentration

The Lineweaver-Burk plot is considered to be more accurate than the Monod plot, since the latter requires an asymptotic determination of the maximum rate of production. Figure (16) shows the relevant Lineweaver - Burk plots. We can immediately conclude that only the glucose fermentation can be described by the Michaelis-Menten model. Only glucose data appear as a straight line. The values for V and K_m for the glucose fermentation were found to be:

$V = 0.396\ hr^{-1}$

$K_m = 9.15\ g/l$

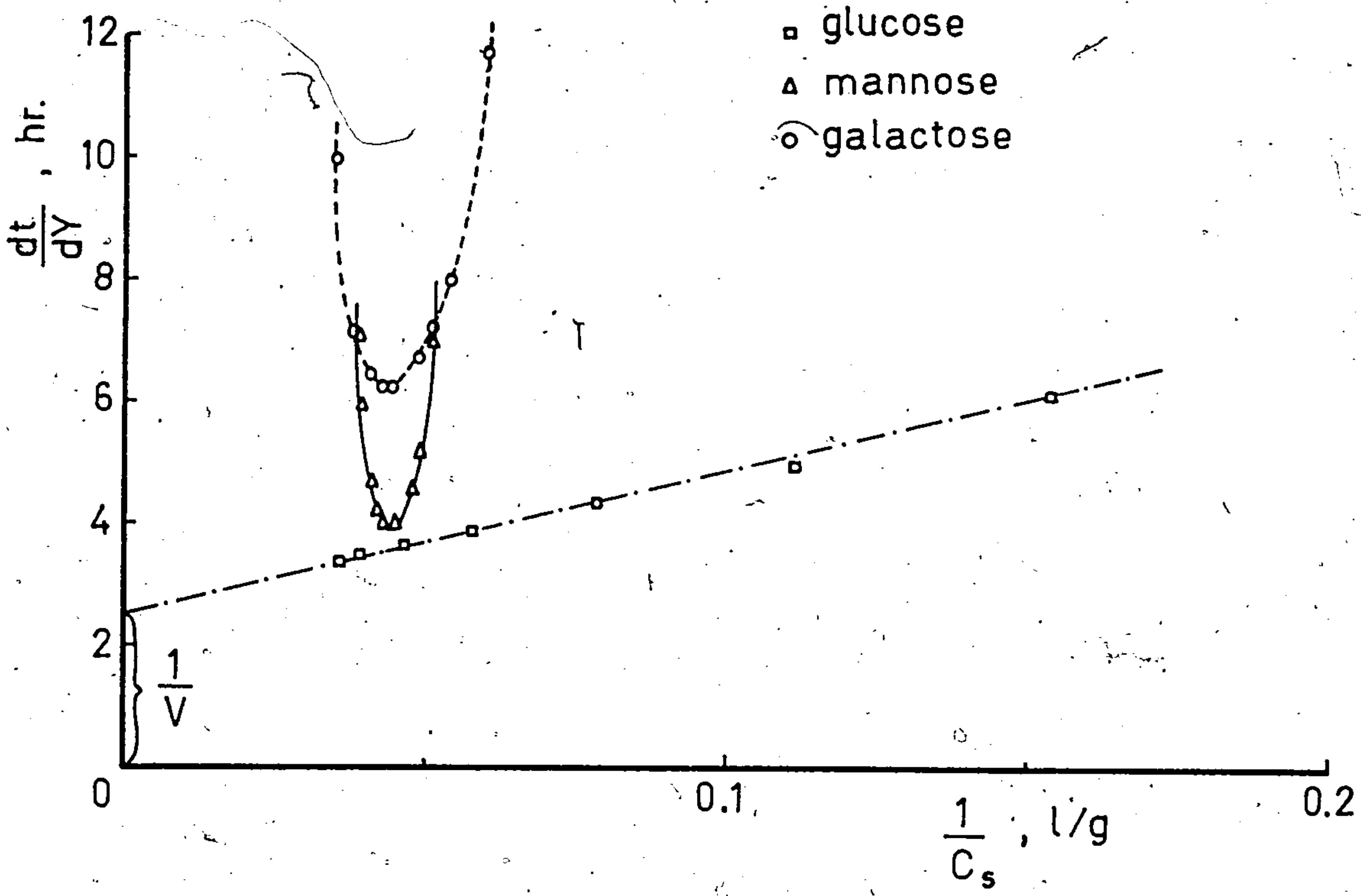

Figure 16 Lineweaver-Burk plot

b. Kono-Asai model:

As mentined before, we were not able to measure the curves for ethanol production. We therefore decided to use a relationship between $-\frac{d C_S}{dt}$ and Y instead of $\frac{d C_p}{dt}$ and Y as required by Equation (7). The previously discussed values for the slopes kp_1 and kp_2 of the triangle -like graph representing a plot of $\frac{d C_p}{dt}$ versus Y could be obtained analytically using the material balance given by Equation (12)

$$- \frac{d C_S}{dt} = f_1 \frac{d C_p}{dt} + f_2 \frac{d Y}{dt} \qquad (12)$$

In the case of the glucose fermentations where f_1 = 1.565 g sugar/ml alcohol and f_2 = 0.94 g sugar/g yeast, kp_1 and kp_2 were found to be positive, i.e. product formation was associated with growth and nongrowth. For the mannose fermentations, with f_1 = 1.565 and f_2 = 1.05, kp_1 was found to be negative and kp_2 was found to be positive; i.e. product formation was decreased with growth and associated with nongrowth. A similar result was obtained for the galactose fermentations. From this it was concluded that this model did not apply for the galactose and mannose fermentations. This could be expected, since there were no exponential growth phases for the relations between - C_S and t, and the model requires this exponential growth phase to be valid.

c. Proposed model for galactose and mannose fermentations:

Representing the obtained data for the galactose and mannose fermentations of Fig. 14 and 15, as shown in Figure 17, where t_l and t_s are respectively t - lag and t - stationary, enables

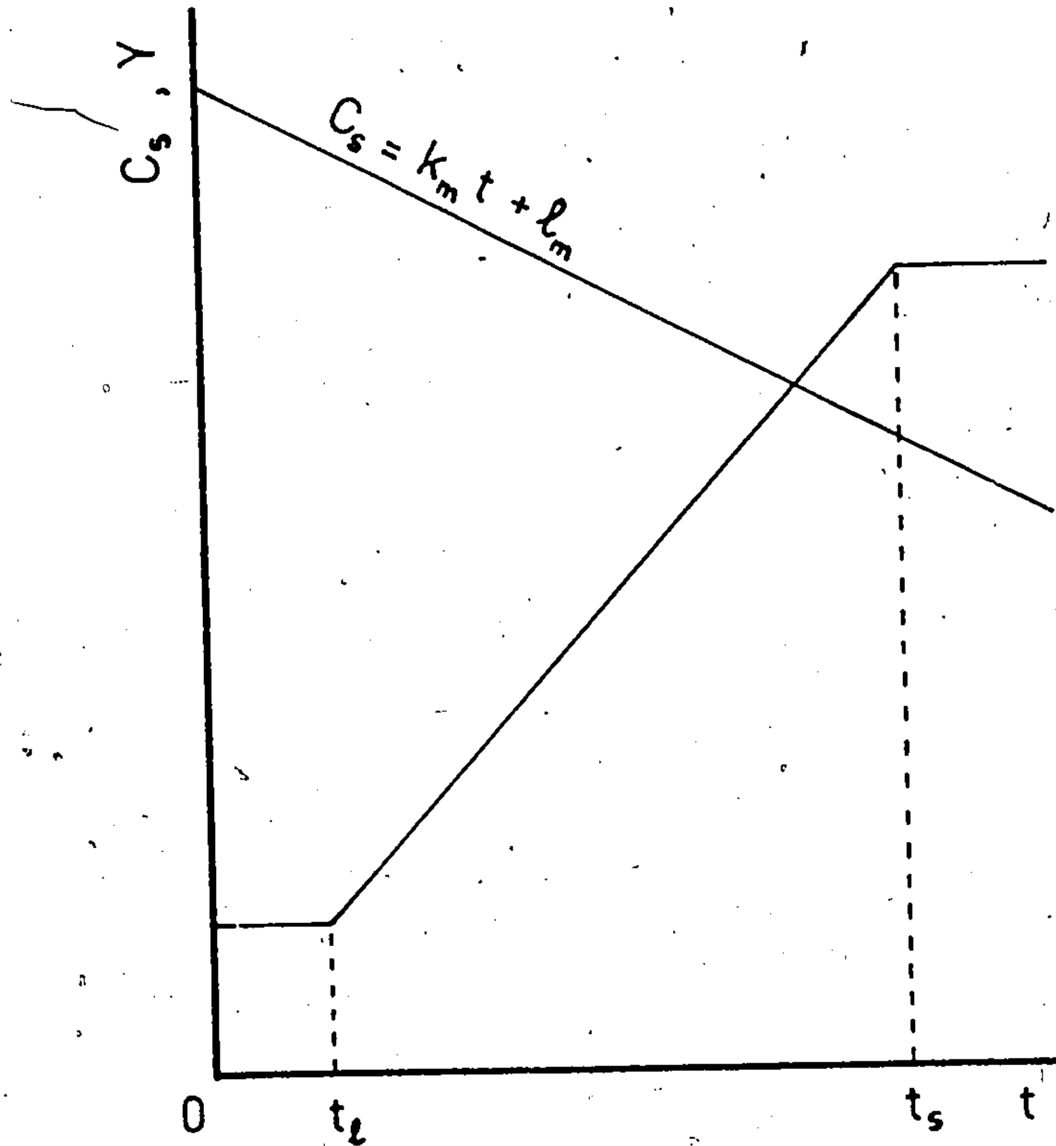

Figure 17 Schematic representation of galactose and mannose fermentations

us to propose a new model to represent these fermentations. By observing that

$$\frac{d\,C_S}{dt} = \text{constant} \tag{13}$$

$$\frac{d\,Y}{dt} = \text{constant} \tag{14}$$

and integrating, we obtained

$$C_S = k_m t + k_n \tag{15}$$

$$Y = a_m t + a_n \tag{16}$$

where k_m, k_n, a_m and a_n are constants. There respective numerical values were determined from the experimental data and are shown in Table 6.

TABLE 6: Observed numerical values for the constants of the model representing the galactose and mannose fermentations.

Sugar	t	k_m	k_n	a_m	a_n
galactose	$0 \leq t \leq t_1$	-1.59	31.5	0	0.4
	$t_1 \leq t \leq t_s$	-1.59	31.5	0.143	0.18
	$t \geq t_s$	-1.59	31.5	0	1.4
mannose	$0 \leq t \leq t_1$	-1.96	31.8	0	0.4
	$t_1 \leq t \leq t_s$	-1.96	31.8	0.184	0.12
	$t \geq t_s$	-1.96	31.8	0	1.6

CONCLUSION

The statistical analysis of the results of the glucose fermentations, investigating the effect of the variables temperature, pH and rpm on the yield of ethanol, for the respective ranges of 27-32°C, 4.0-6.0 and 500-700, combined with the results of the work of Aiyar and Luedeking [26] enabled us to conclude that the best set of conditions to produce ethanol from glucose by Saccharomyces Cerevisiae is:

- temperature: 32°C
- pH: 5.0
- rpm: 700

The conditions were also applied to the fermentation of galactose and mannose, which form about 85% of the hexose concentration of softwoods. Complete sugar utilization yielded a measured ethanol concentration of 17.9 ml/l in the case of the glucose fermentation, using a 33.3 g/l sugar solution. For the galactose and mannose fermentations sugar utilization was incomplete during the ten hour fermentation period. The ethanol concentration for zero sugar concentration could however be estimated to be about 10 ml/l and 15 ml/l for the galactose and mannose fermentations respectively. The kinetic models of Michaelis-Menten and of Kono and Asai were found to describe only the glucose fermentation. A model was proposed to describe the galactose and mannose fermentations.

Recommendations for further work

This work must be considered preliminary and much more experimentation is necessary before the ultimate utility of this fermentation can be assessed. Work should be continued with particular emphasis on:

- investigation of effects of factors such as aeration, agitation and medium composition, for the purpose of increasing fermentation rate.
- investigation of the fermentation of all three sugars together.
- investigation of economical procedures for recovering the products.
- investigation of continuous fermentation

NOMENCLATURE

Symbol	Definition	Units
a_m	constant	g/lhr
a_n	constant	g/l
b_S	constant for sugar	l/g
C_o	oxygen concentration	g/l
C_S	sugar concentration	g/l
C_P	product concentration	ml/l
E	effect of variable	
k	number of variables	
k_m	constant	g/lhr
K_m	Michaelis-Menten constant	g/l
k_n	constant	g/l
kp_1	production rate constant	hr^{-1}
kp_2	production rate constant	hr^{-1}
t	fermentation time	hr
v	rate of product formation	hr^{-1}
V	maximum rate of production	hr^{-1}
x	variable	
Y	cell concentration	
ϕ	apparent coefficient of growth activity	
σ	standard deviation	

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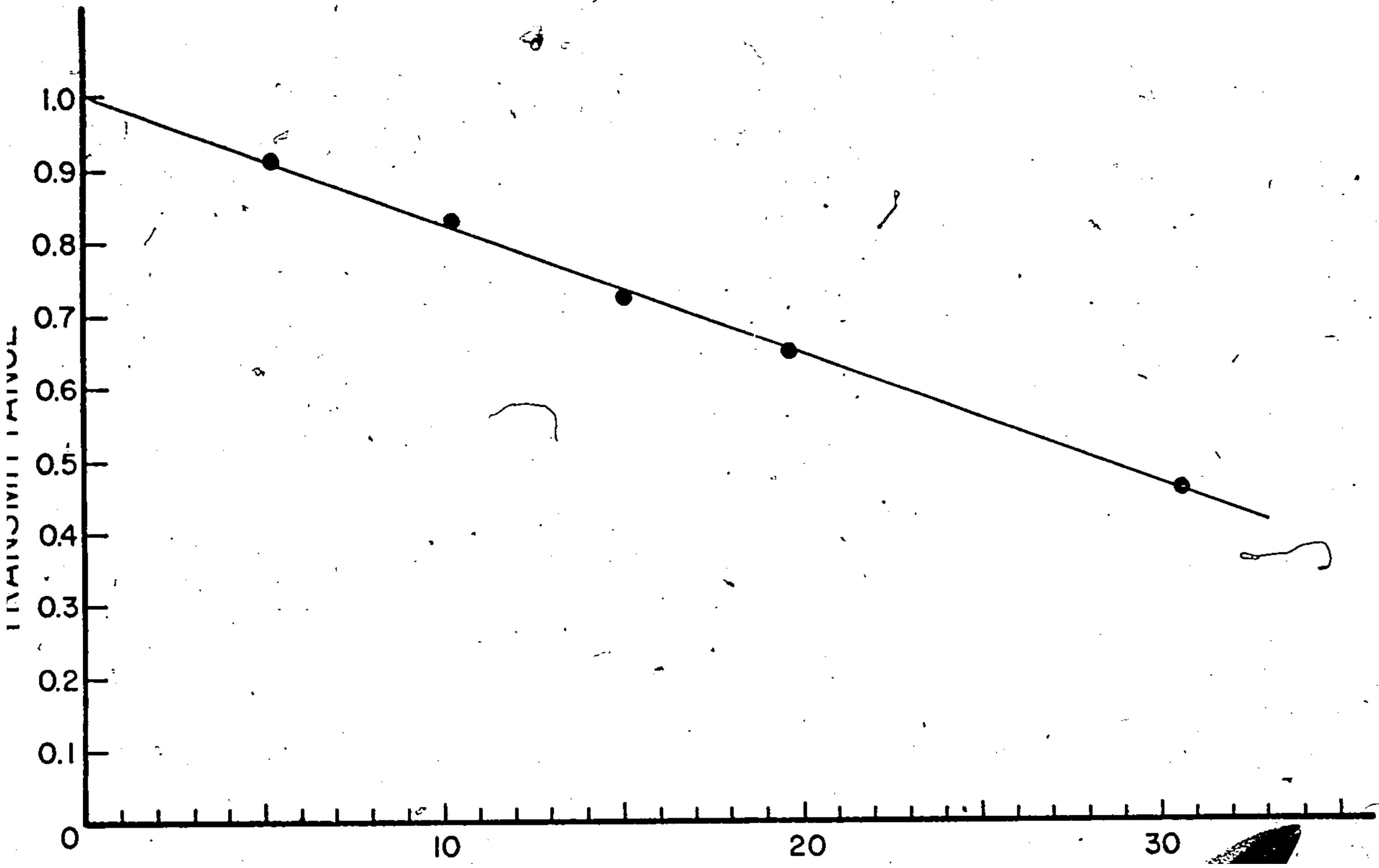
APPENDICES

1. Composition of 100 g. of yeast nitrogen base as supplied by DIFCO laboratories

Copper sulphate	589 mcg
Biotin	29 mcg
Folic acid	29 mcg
Boric acid	7.365 mg
Potassium iodide	1.473 mg
Ferric chloride	2.946 mg
Manganese sulfate	5.892 mg
Sodium molybdate	2.946 mg
Zinc sulfate	5.892 mg
Calcium pantothenate	5.892 mg
Inositol	29.460 mg
Niacin	5.892 mg
p-Aminobenzoic acid	2.946 mg
Pyocydoxine Hydrochloride	5.892 mg
Riboflavin	2.946 mg
Thiamine Hydrochloride	5.892 mg
L-Histidine Monohydrochloride	147.318 mg
DL - Methionine	294.636 mg
DL - Tryptophane	294.636 mg
Magnesium sulfate	7.365 g
Sodium chloride	1.473 g
Calcium chloride	1.473 g
Ammonium sulfate	73.650 g
Monopotassium phosphate	14.732 g

2. Calibration curves for the spectrophotometric analysis of dextrose (Fig. 5), galactose (Fig. 6) mannose (Fig. 7) and for the gas chromatographic analysis of ethanol (Fig. 8).

Figure 5 Calibration curve for spectrophotometric dextrose analysis



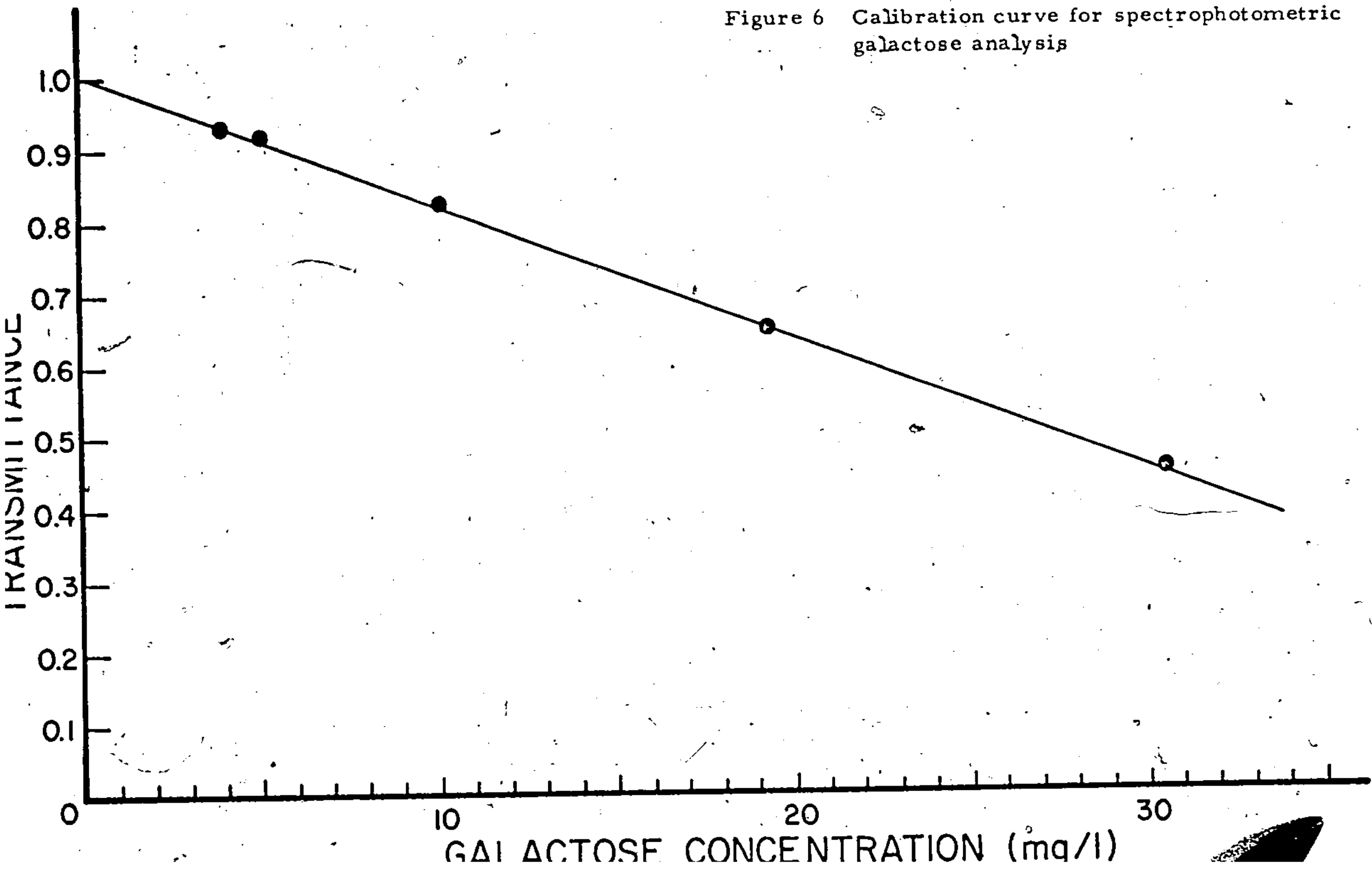


Figure 6 Calibration curve for spectrophotometric galactose analysis

Figure 7 Calibration curve for spectrophotometric mannose analysis

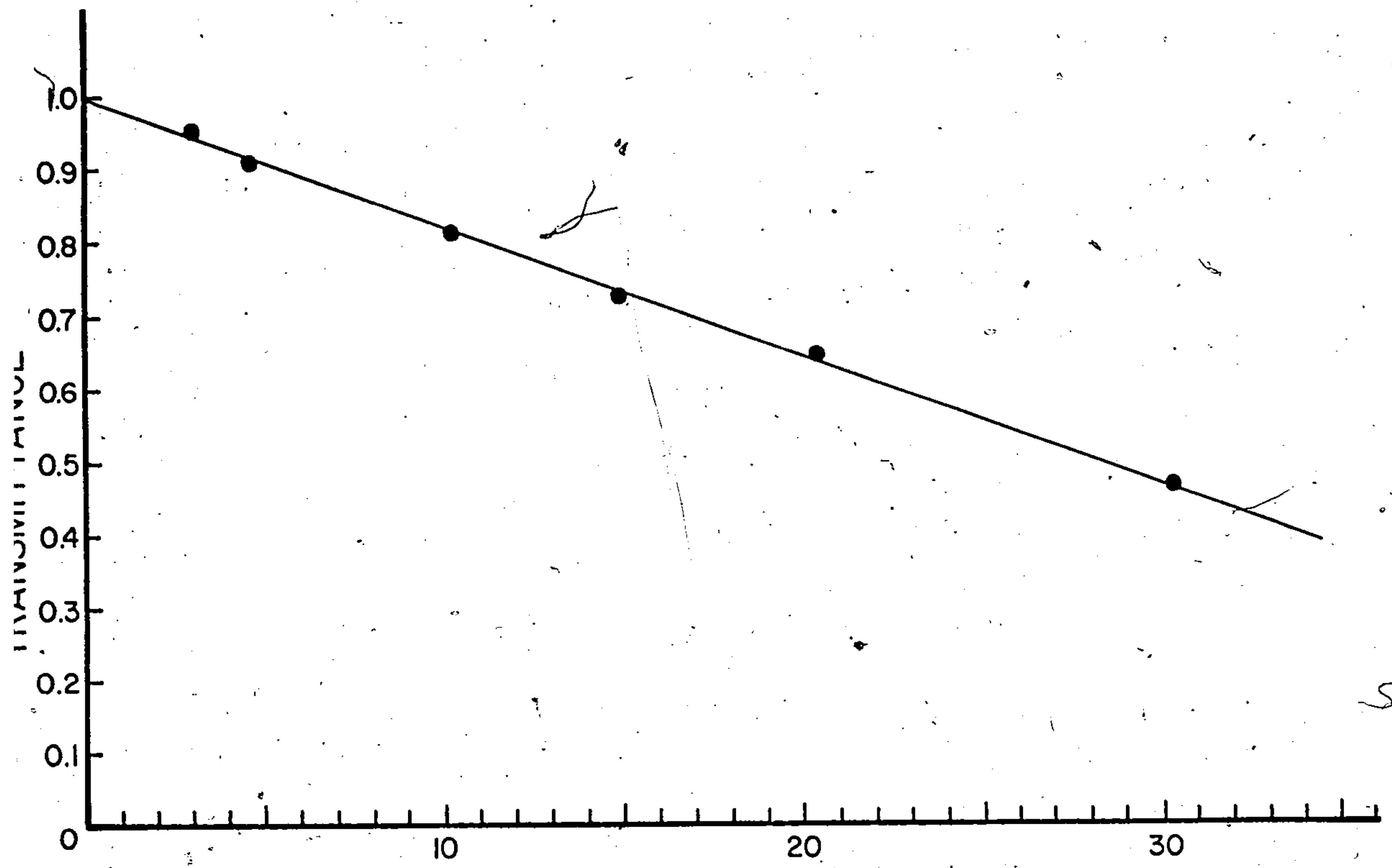
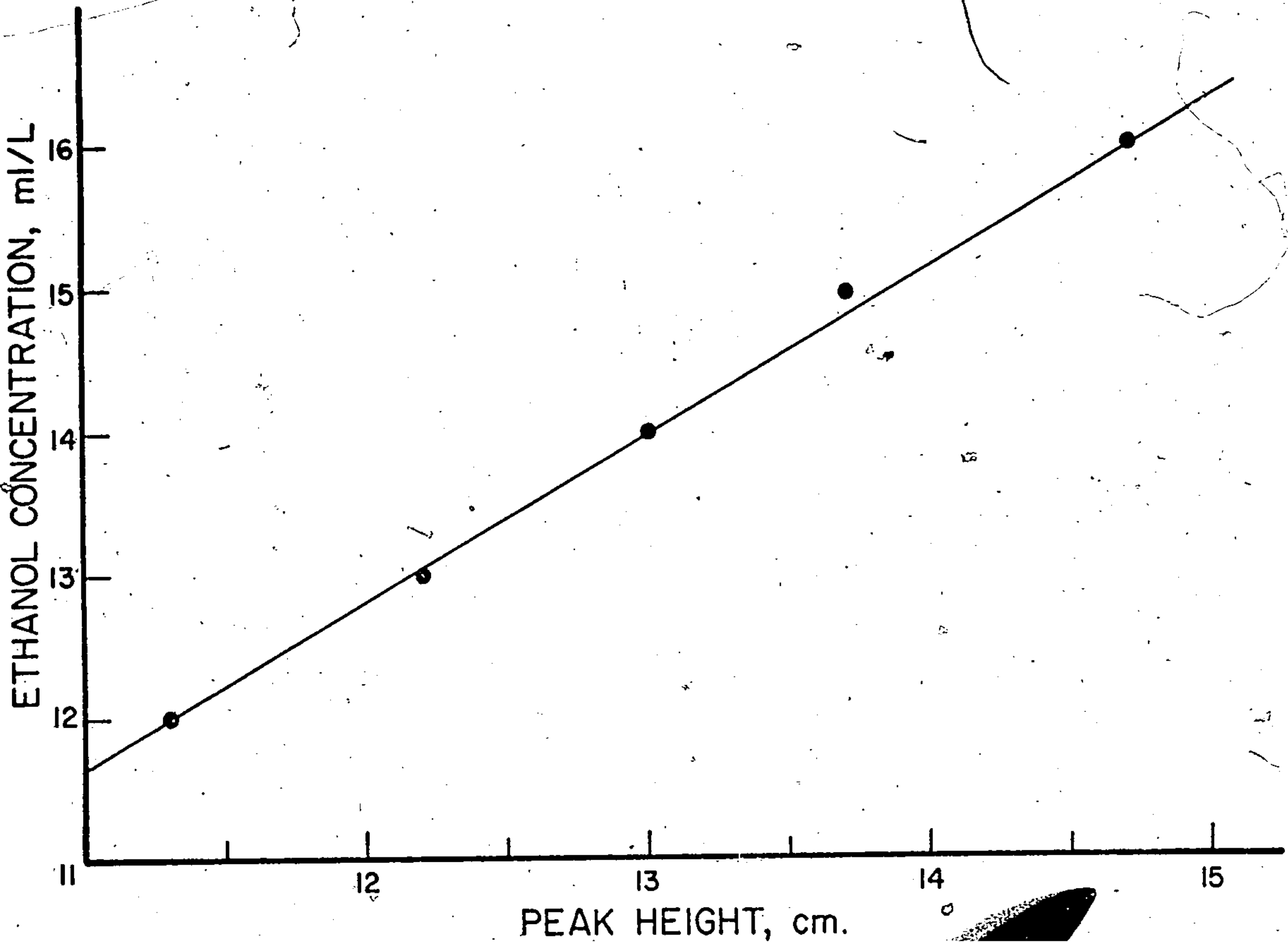


Figure 8 Calibration curve for chromatographic ethanol analysis



3. Results for part 1

Run No. 1
Y.N.B. 13.3 g/l
C° glucose 33.3 g/l
Y_o^s 1 33 g/l

T° = 29°C
pH = 5.0
rpm = 400
Q = 7000 cc/min

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	1	0.3	40.5	33.4	
2	2	0.5	42.6	32.3	
3	3	0.8	39.6	33.8	
4	4.15	1.1	46.5	30.0	
5	5	1.9	54.1	25.8	
6	6	2.1	68.0	18.0	
7	7	2.5	88.4	6.8	
8	8	2.9	92.3	4.5	
9	9	3.0	95.7	2.6	14.2
10	10	3.1	100	0	14.1
11	11	3.0	100	0	

Run No. 2

Y.N.B. 13.3 g/l

C°_s glucose 33.3 g/l

Y° 1.33 g/l

T° = 29°C

pH = 5.0

rpm = 400

Ω = 7000 cc/min

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	40.5	33.4	
2	1	0.4	41.6	32.8	
3	2	0.5	43.6	31.9	
4	3.15	0.8	46.5	30.0	
5	4	0.9	64.2	20.2	
6	5.50	1.9	78.1	12.4	
7	6.50	2.4	88.7	6.5	
8	7.50	2.9	93.2	4.0	14.8
9	8.50	3.0	100	0	13.4
10	9.50	3.0	100	0	16.4

Run No. 3

Y.N.B. 13.3 g/l

C°_s glucose 33.3 g/l

Y° 1.33 g/l

T° = 29°C

pH = 5.0

rpm = 400

Q = 7000 cc/min

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	40.5	33.4	
2	0.75	0.4	42	32.6	
3	1.75	0.5	43.5	31.8	
4	2.75	0.7	46.5	30.0	
5	3.75	1.0	42.0	32.6	
6	4.75	1.6	68.0	18.0	
7	5.75	2.0	90.5	6.0	
8	6.75	2.8	95.0	3.0	
9	7.75	2.9	100	0	12.7
10	8.75	2.9	100	0	14.8
11	9.50	3.0	100	0	14.0

4. Results of part 2

Run No. 4 | T° = 32°C
Y.N.B. 13.3 g/l | pH = 6.0
C°_{s} glucose 33.3 g/l | rpm = 700
Y° 1.33 g/l | Q = 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.4	31.5	38.3	
2	0.75	0.5	35	36.4	
3	1.50	0.7	39	34.2	
4	3.50	1.8	37.5	35	
5	4.50	2.3	68.5	17.8	
6	5.50	2.8	82.5	10	
7	6.50	2.8	99.0	0.8	13.3
8	7.50	3.0	100	0	16.7
9	8.25	2.9	100	0	16.8

Run No. 5 | T° = 27°C
Y.N.B. 13.3 g/l | pH = 4.0
C°_{s} glucose 33.3 g/l | rpm = 500
Y° 1.33 g/l | Q = 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	41	33.2	
2	1	0.4	42.5	32.2	
3	2	0.7	48.5	29	
4	3	1.0	56	24.8	
5	4	1.6	67.5	18.4	
6	5.33	2.5	82.5	10	
7	6.15	2.7	92	4.8	
8	7	3.0	98.5	1	15.6
9	7.75	3.0	99.5	0.5	15.4
10	8.50	3.0	100	0	14

Run No. 6
Y.N.B. 13.3 g/l
C°_{s} glucose 33.3 g/l
Y° 1.33 g/l

T° = 27°C
pH = 4.0
rpm = 500
Q = 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	44	31.5	
2	1	0.5	45	31	
3	2	0.6	46.5	30	
4	3	0.8	63.5	20.6	
5	4	1.3	56.5	24.5	
6	5	1.8	75.5	14	
7	6.15	2.0	80.5	11.2	
8	7.30	2.3	100	0	
9	8	2.5	100	0	14.7
10	9.20	2.7	100	0	16.8
11	10.20	2.6	100	0	17.2

Run No. 7
Y.N.B. 13.3 g/l
C°_{s} glucose 33.3 g/l
Y° 1.33 g/l

T° = 32°C
pH = 4.0
rpm = 500
Q = 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	39	34.2	
2	1	0.5	41.5	33.0	
3	2.50	0.9	43.5	31.7	
4	3.50	1.5	54	25.8	
5	4.50	1.9	62.5	21.2	
6	5.65	2.2	78	12.6	
7	6.75	2.3	92.5	4.5	14.9
8	8	2.5	100	0	
9	9	2.1	100	0	14.8

Run N° 8
Y.N.B. 13.3 g/l
C°$_s$ glucose 33.3 g/l
Y°^{s} 1.33 g/l

T° 32°C
pH 4.0
rpm 500
Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	57.5	24	
2	1	0.5	55.5	25	
3	2	1.0	56.6	24.6	
4	3	1.4	64.5	20	
5	4	2.0	61	22	
6	5.85	2.5	88.5	6.7	14.6
7	6.75	2.5	97	2	15.8
8	7.75	2.1	100	0	15.8

Run N° 9
Y.N.B. 13.3 g/l
C°$_s$ glucose 33.3 g/l
Y°^{s} 1.33 g/l

T° 27°C
pH 4.0
rpm 700
Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	40.5	33.4	
2	1.50	0.5	41.5	33.0	
3	3	0.7	43.5	31.7	
4	3.75	0.9	47.5	29.5	
5	4.75	1.1	57.0	24.2	
6	5.75	1.6	71.5	16.2	
7	6.75	2.1	73.5	15.0	
8	8.75	1.9	100	0	12.9
9	9.75	1.8	100	0	14.7

Run N° 10
Y. N. B. 13.3 g/l
C°_{s} glucose 33.3 g/l
Y°_{s} 1.33 g/l

T° 27°C
ph 4.0
rpm 700
Q 7000 cc/min for 4.50 hr

Sample No	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.4	39.5	34	
2	1.15	0.4	42.5	32.3	
3	2.75	0.6	48	29.2	
4	4	1.0	51.5	27.4	
5	5	1.2	63.5	20.6	
6	6	1.5	81	11	
7	7.50	2.0	100	0	
8	8.50	1.9	99.8	0.4	10.1
9	9.65	2.0	100	0	16.5

Run N° 11
Y.N.B. 13.3 g/l
C°_{s} glucose 33.3 g/l
Y°_{s} 1.33 g/l

T° 32°C
pH 6.0
rpm 500
Q 7000 cc/min for 4.50 hr

Sample No	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3			
2	1.33	0.8	43	32.0	
3	2.40	1.0	48.5	29	
4	3.40	1.3	61.5	21.7	
5	4.40	1.8	76	13.8	
6	5.65	2.0	86.5	7.8	
7	6.65	2.0	95.5	2.7	13.2
8	8.30	2.4	98	1.4	13.7
9	9.15	2.2	100	0	13.1

Run No. 14 T° 27°C
Y.N.B. 13.3 g/l pH 6.0
C°_{s} glucose 33.3 g/l rpm 500
Y° 1.33 g/l Q 7000 cc/min for 4.50 hr

Sample N°	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.3	42	32.6	
2	1	0.4	48	29.2	
3	2	0.5	46.5	30	
4	3	0.6	55	25.4	
5	4	1.2	58.5	23.4	
6	5.50	1.5	71.5	16.2	
7	6.75	2.0	80	11.4	
8	7.60	2.1	93	4	7
9	8.50	2.2	100	0	13.2

Run No. 15 T° 32°C
Y.N.B. 13.3 g/l pH 4.0
C°_{s} glucose 33.3 g/l rpm 700
Y° 1.33 g/l Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.4	78.5	12.4	
2	1.50	0.5	81	11.0	
3	3.40	1.0	83	9.8	
4	4.40	1.7	95.5	2.8	
5	5.50	2.0	99.5	0.5	9.2
6	6.50	2.1	100	0	14.7
7	7.40	2.1	100	0	18.8

Run No. 16 | T° 32°C
Y.N.B. 13.3 g/l | pH 4.0
C°_s glucose 33.3 g/l | rpm 700
Y°_s 1.33 g/l | Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.4			
2	1.25	0.6	45	31	
3	2.75	1.0	51.5	27.3	
4	4.50	1.7	64	20.3	
5	5.50	2.0	78	12.6	
6	6.35	2.2	91.5	5.0	14.9
7	7.30	2.2	99.5	0.6	16.4
8	8.75	2.0	100	0	16.4

Run No. 17 | T° 37°C
Y.N.B. 13.3 g/l | pH 6.0
C°_s glucose 33.3 g/l | rpm 700
Y°_s 1.33 g/l | Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	1.15	0.5	48.5	29	
2	2.50	1.0	54	26	
3	4.50	1.8	70	17	
4	6.50	2.0	-	-	14.1
5	7.50	2.0	100	0	15.6
6	9.50	1.9	100	0	15.2

Run No. 18
Y.N.B. 13.3 g/l
C°_s glucose 33.3 g/l
Y° 1.33 g/l
T° 27°C
pH 6.0
rpm 700
Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0.50	0.35	39	34.2	
2	2	0.5	-	-	
3	4	1.2	44.5	31.2	
4	5	1.6	52	27	
5	6.50	2.0	70.5	17.8	9.9
6	8	2.1	99.5	0.5	15.3
7	9.50	2.0	100	0	18.3

Run No. 19
Y.N.B. 13.3 g/l
C°_s glucose 33.3 g/l
Y° 1.33 g/l
T° 27°C
pH 6.0
rpm 700
Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0.75	0.4	38.5	34.5	
2	2.25	0.7	43	32	
3	3.75	1.0	42.5	32.4	
4	5.25	1.6	54	26	
5	6.75	1.9	76	13.8	8.7
6	8.75	2.0	99.5	0.6	13.5
7	9.75	2.0	100	0	15.6

5. Results of part 3

Run No. 20 — T° 32°C
Y.N.B. 13.3 g/l — pH 5.0
C°_s glucose 33.3 g/l — rpm 700
Y° 1.33 g/l — Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0.25	0.3	38.5	34.5	
2	1.25	0.5	36	35.8	
3	3.25	1.0	42.5	32.3	
4	4.25	1.4	60.4	22.3	
5	5.50	1.8	74.5	14.5	
6	7.50	2.3	99.5	0.5	16.9
7	8.75	2.1	100	0	19.4
8	10.25	2.1	100	0	15.6

Run No. 21 — T° 32°C
Y.N.B. 13.3 g/l — pH 5.0
C°_s glucose 33.3 g/l — rpm 700
Y° 1.33 g/l — Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	1	0.5	39.5	34	
2	2.50	0.8	37.5	35.2	
3	4	1.1	42.5	32.3	
4	5.50	1.8	59.5	23	
5	7	2.1	88	7	19.7
6	8.50	1.8	99.5	0.5	18.5
7	10	2.0	100	0	20.2

Run No. 22 T° 32°C
Y.N.B. 13.3 g/l pH 5.0
C°_{s} mannose 33.3 g/l rpm 700
Y° 1.33 g/l Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0.75	0.4	47	30	
2	2.25	0.6	50	28.2	
3	3.75	0.7	59.5	23	
4	5.25	0.8	60.5	22.6	
5	6.75	1.4	63.5	21	
6	8.25	1.5	-	-	6.5
7	9.75	1.7	71.5	16.3	9.5

Run No. 23 T° 32°C
Y.N.B. 13.3 g/l pH 5.0
C°_{s} mannose 33.3 g/l rpm 700
Y° 1.33 g/l Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.4	44	31.6	
2	1.50	0.4	48	29.6	
3	3	0.7	61.5	22	
4	4.50	0.9	58.5	23.8	
5	6	1.2	61	22.3	
6	7.50	1.6	67	18.8	5.8
7	10.25	1.8	74	15	9

Run No. 24 — T° 32°C
Y.N.B. 13.3 g/l — pH 5.0
C°_s galactose 33.3 g/l — rpm 700
Y° 1.33 g/l — Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0	0.4	41.5	31	
2	1.50	0.4	45	30	
3	3	0.6	53.5	25.4	
4	4.50	1.0	65	19.2	
5	6	1.1	64.5	19.5	
6	8.25	1.3	68	17.6	3.8
7	10.25	1.4	74.5	14	8.5

Run No. 25 — T° 32°C
Y.N.B. 13.3 g/l — pH 5.0
C°_s galactose 33.3 g/l — rpm 700
Y° 1.33 g/l — Q 7000 cc/min for 4.50 hr

Sample No.	Time (hr)	Yeast concentration (vol. %)	Transmittance (490 nm)	Sugar concentration (g/l)	Alcohol concentration (ml/l)
1	0.50	0.4	44	30.6	
2	1.50	0.4	45.5	29.8	
3	3	0.5	61.5	21.6	
4	4.50	0.8	56	24	
5	6.50	1.0	67	18	
6	8.50	1.4	76.5	13	5.9
7	10	1.3	78	12	7.6

6. CALCULATION EXAMPLES

Although the cubes method is practical for the average effect as well as for the two-factor interactions, this method becomes impossible to use if applied to more than three variables. We shall therefore use a calculation procedure that is applied to the analysis of two-level factorial designs involving any number of variables. We first construct a calculation matrix from the design matrix in the following manner:

Calculation Matrix

Design Matrix

x_1	x_2	x_3	x_{12}	x_{13}	x_{23}	x_{123}	y
-1	-1	-1	+1	+1	+1	-1	14
+1	-1	-1	-1	-1	+1	+1	12.9
-1	+1	-1	-1	+1	-1	+1	15.3
+1	+1	-1	+1	-1	-1	-1	14.3
-1	-1	+1	+1	-1	-1	+1	12.6
+1	-1	+1	-1	+1	-1	-1	15
-1	+1	+1	-1	-1	+1	-1	15.4
+1	+1	+1	+1	+1	+1	+1	16.1

Now the average effects and interactions are obtained by multiplying the relevant column of effect (x_1, x_2, x_3) or interaction (x_{12}, x_{13}, x_{23}) by the y column, and then dividing by 4. For the average effect of pH : E_1 we then have

$$\underbrace{\begin{bmatrix} -1 \\ +1 \\ -1 \\ +1 \\ -1 \\ +1 \\ -1 \\ +1 \end{bmatrix}}_{x_1} \quad X \quad \underbrace{\begin{bmatrix} 14 \\ 12.9 \\ 15.3 \\ 14.3 \\ 12.6 \\ 15 \\ 15.4 \\ 16.1 \end{bmatrix}}_{y} = \begin{bmatrix} -14 \\ +12.9 \\ -15.3 \\ +14.3 \\ -12.6 \\ +15 \\ -15.4 \\ +16.1 \end{bmatrix}$$

$$\Sigma = 1$$

$$\text{so } E_1 = \Sigma/4 = 0.25$$

For the average interaction E_{123} we find:

$$\underbrace{\begin{bmatrix} -1 \\ +1 \\ +1 \\ -1 \\ +1 \\ -1 \\ -1 \\ +1 \end{bmatrix}}_{x_{123}} \quad X \quad \underbrace{\begin{bmatrix} 14 \\ 12.9 \\ 15.3 \\ 14.3 \\ 12.6 \\ 15 \\ 15.4 \\ 16.1 \end{bmatrix}}_{y} = \begin{bmatrix} -14 \\ +12.9 \\ +15.3 \\ -14.3 \\ +12.6 \\ -15 \\ -15.4 \\ +16.1 \end{bmatrix}$$

$$\Sigma = -1.8$$

$$\text{and } E_{123} = \frac{-1.8}{4} = -0.45$$

Other effects and interactions can be computed in a similar way.

Summarizing the obtained results we have:

1. average effects $E_1 = 0.25$ (pH)
   $E_2 = 1.65$ (T°)
   $E_3 = 0.65$ (rpm)
2. Two-factor interaction $E_{12} = -0.40$
   $E_{13} = 1.30$
   $E_{23} = 0.30$
3. Three-factor interaction $E_{123} = -0.45$
4. average y = 14.45 with $\sigma = 1.23$

   where $\sigma^2 = \frac{1}{n-1} \Sigma (y_i - \bar{y})^2$

Other effects and interactions can be computed in a similar way.

Summarizing the obtained results we have:

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   where $\sigma^2 = \frac{1}{n-1} \Sigma (y_i - \bar{y})^2$