STUDIES IN THE CONTINUOUS TOWER FERMENTATION OF ALCOHOL

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A thesis submitted to the University of Aston in Birmingham in fulfilment of the requirements for the degree of Doctor of Philosophy.

October, 1974.

Certificate

I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

Signed ..... S. Coote .....

Candidate

Date ..... 4 Nov 1974 .....

I hereby declare that the whole of the work now submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.

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Candidate

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Supervisor

Acknowledgements

I wish to express my appreciation to the following persons for their various assistance.

Dr. R. N. Greenshields for the supervision and guidance which I have received during the course of this work.

Mr. D. T. Shore and the Directors of the A. P. V. Co. Ltd., for providing a research studentship.

Mrs. D. Daunter for her patience and skill in typing this thesis.

My colleagues in the Department of Biological Sciences and Chemical Engineering.

Contents

List of Figures

List of Plates

SUMMARY

The introduction of continuous tower fermentation for the production of beer and industrial alcohol has posed the questions; which type of yeast is most suited to the system and what conditions produce maximum performance? This study is an attempt to provide some answers.

A method of selecting yeasts was devised based upon a sedimentation test and assessment in a laboratory tower fermenter, designed to simulate an industrial unit.

Pure culture yeasts of the species Saccharomyces cerevisiae, Saccharomyces uvarum and Saccharomyces diastaticus were cultivated in a tower fermenter. From this work, cultures of each were selected as suitable for further investigation.

It has been suggested that the prolonged continuous cultivation of yeast can result in undesirable changes which are due to mutation. Studies were made to determine whether mutation would present a serious hazard in continuous tower fermentation.

Fifty cultures of a brewery yeast (Saccharomyces cerevisiae) were isolated before and after 134 days' continuous fermentation in a laboratory tower fermenter. A decrease in flocculence was was noted in two isolates but the population as a whole showed an increase in flocculence. A change in giant colony morphology was noted in two isolates but no changes were noted in any of the other characteristics examined, i.e. cell shape, spore formation, melibiose fermentation.

Similar and additional tests (fermentation rate, yeast yield and attenuation gravity) were applied to the same strain of yeast which had been cultivated in a production scale fermenter for one year. Five isolates showed unusual giant colony characteristics

and a slight decrease in flocculence was noted for the population as a whole. Eight isolates failed to produce ascospores. There were no changes in other characteristics. Because of the limited nature of the changes, it was concluded that mutation does not present a serious practical hazard to the operation of a tower fermenter for prolonged periods.

The continuous tower fermentation of lager beer has been complicated by the presence of high concentrations of vicinal diketones in the product. These compounds are normally found in beer in concentrations up to 0.1 ppm but above 0.2 ppm the flavour of the beer is impaired. Fourteen strains of lager yeasts were assessed against criteria of flocculence and amounts of vicinal diketones in the finished beer. Four yeasts were tested in the laboratory tower fermenter and of these two were found to be suitable for further investigation.

The selected cultures were cultivated in the fermenter and operational parameters of wort flow rate, temperature and aeration altered to determine the most suitable conditions.

It was concluded that the fermenter must be operated at slow wort flow rates with a relatively high temperature in the top half of the fermenter.

Previously the tower fermenter has not been applied to the continuous fermentation of molasses mashes for industrial alcohol.

A strain of Saccharomyces diastaticus was selected and operated in the tower fermenter. It was shown that mashes containing up to 20 g% w/v sugar could be successfully fermented. The parameters influencing the rate of fermentation were investigated. It was concluded that the production of industrial alcohol is an application in which the tower fermenter may be

successfully employed.

The value of this work in relation to industrial requirements and the role of the laboratory fermenter in assessing flocculence has been discussed.

INTRODUCTION

i. Historical Introduction

No date can be assigned to the first observation of the phenomenon of alcoholic fermentation but the mention of fermentation can be found in the earliest chemical writings. Throughout the period of alchemy the process of fermentation played an important part, and many ideas were based on fermentation. The distinction between fermentation and other processes in which gases were evolved, such as the action of acids on carbonates, dates from the mid-17th century (Harden, 1911) and soon it was realised only sweet liquors could be fermented. The first definite ideas about fermentation were proposed by Stahl (1697) but it was not until Lavoisier (1789) analysed the chemicals involved in fermentation, that any clear idea of the chemical changes occurring was formed.

In 1851 Pasteur began his researches which led him to the conclusion that "alcoholic fermentation is an act correlated with the life and organisation of the yeast cells". Subsequently Pasteur came to the conclusion that alcoholic fermentation was the result of life without oxygen.

For some time there has been an opposing school of thought which, headed by Liebig, believed that fermentation was due to some chemical substance produced by the yeast cell. In 1858 Traube had published a theory which proposed that all fermentation was caused by definite chemical substances, which were related to proteins. The successful preparations of invertase by Berthelot (1860) lent support to the enzyme theory of fermentation, but many attempts by various workers to extract the substances responsible for fermentation failed. It was not until 1897 that Eduard Buchner succeeded in preparing an

extract from yeast which would ferment sugar to alcohol without the presence of intact yeast cells. Buchner's discovery placed the study of fermentation on a new level and research methods for the elucidation of the fermentation process greatly benefitted such that within a few years Harden and Young (1905) and Buchner and Haehn (1910) had established that phosphate and a co-enzyme played an important role in fermentation.

The increasing knowledge of the processes involved in fermentation together with the establishment of yeast as the organism responsible, provided the stimulus for development in the brewing industry. In 1883, Hansen suggested the use of pure strain culture yeast. Traditional practice in the brewing industry involved using a batch fermentation process, and some differences in techniques between British and Continental practice had been established, each giving a characteristic type of beer.

ii. Ale beer

Brewing in the British style has involved utilizing a strain of Saccharomyces cerevisiae, commonly called a top fermenting yeast, because of the formation of a yeasty head on the beer during and at the end of fermentation. The yeast could be removed by suction or skimming (the skimming system), but if, during the fermentation, the beer was fed to another vessel by gravity the method was called the dropping system. The wort was fed, from above into open vessels, two to four metres deep, and pitched with yeast as soon as possible. The temperature at the start of fermentation was normally around 15^{o}C but was allowed to rise to 20^{o}C as the fermentation proceeded. In the past mixing and aeration was achieved by 'rousing' with a paddle but nowadays the wort is circulated through an electric pump and sprayed

back on to the surface of the beer. In the dropping system sufficient mixing occured when the fermenting wort was transferred to another vessel. The yeast was removed from the head of the beer up to three times during fermentation and when the attenuation of the beer was satisfactory the yeast on the head of the beer was removed by skimming. Today separation of the yeast can be facilitated by reducing the temperature to 14 - 15^{o}C at the end of fermentation.

The traditional method of brewing described above is still practised though modern developments have altered the process in detail. Various modifications of the basic techniques are practised in different areas of the country. The development of systems which achieve circulation of the fermenting wort and sedimentation of the yeast at the end of fermentation is illustrated by reference to the Burton Union and the Yorkshire Stone Square systems.

The Burton Union system of fermentation comprises a series of oak casks arranged in two adjacent rows below the main fermentation vessel. The wort is dropped into the casks at the height of fermentation. An inclined trough is positioned above the casks and during active fermentation the yeast and beer rises through pipes to the trough. The yeast sediments out and the beer returns to the casks by pipes which enter the end of the casks. By the end of fermentation most of the yeast has sedimented in the trough and the beer is led into a racking vessel. In some modern versions of this system the oak casks have been replaced by a single stainless steel tank but in all other respects the system is similar.

The fermentation vessels used in the Yorkshire Stone Square system were originally made of stone and constructed in two compartments - an upper and a lower which were separated by a deck. The two compartments were connected by a series of pipes and a central

manhole with a flange 15 cm high. The vessel was filled with wort to a depth of 2.5 cm in the upper compartment. During the fermentation the yeast rose through the manhole and sedimented on the deck, whilst the beer drained back into the lower vessel. Eventually the circulation stopped and the yeast was skimmed off the deck and the beer was racked off from the lower compartment.

One of the primary disadvantages of the traditional fermentation systems was the large amounts of space required to house the vessels which were necessarily shallow to ensure adequate separation of the yeast. The introduction of continuous discharge centrifuges has permitted the adoption of different designs of fermenter which the cylindro-conical vessel is an example of. A typical cylindo-conical vessel of gross capacity 851 hl. would be approximately 10 m high and 3.6 m in diameter. The completely closed construction allows the vessel to be pressurised and has other advantages: 1) easy collection of CO_2; 2) suitable for automatic cleaning; 3) less floor space required for a fermentation of a given volume.

iii. Lager beer

Continental practice has favoured the use of Saccharomyces uvarum as a brewing yeast which typically settled to the bottom of the fermentation vessel, at the end of fermentation.

The traditional lager type of fermentation is still widely practised and is conducted in open vessels which are filled to within 30 cm of the top with the wort. Normally the yeast is premixed with the wort, which enters the vessel gently through a port in the bottom. The fermentation is started at approximately $6^{o}C$ and the temperature is allowed to rise to $10^{o}C$ by the midpoint and then gradually reduced to $5^{o}C$ at completion, on the ninth to twelfth day. The yeast sediments to the base of the vessel and the beer is run gently to lagering tanks.

Traditionally, lagering was carried out in cylindrical tanks at a temperature between 0^{o} and $5^{o}C$ for a period of several months, but with modern techniques the period required for flavour development has been reduced to a few weeks.

The use of cylindro-conical vessels for bottom fermentation was advocated by Nathan (1930). More recently it has been reported that the period of primary fermentation can be subsequently reduced by utilizing this type of vessel (Haboucha et alia, 1970). The vessel was partially filled with wort and then seeded with a large amount of yeast. When the fermentation was nearly complete fresh wort was added continuously at a rate designed to maintain the attenuation near the limit. By use of this technique the fermentation was completed in approximately 48 hours.

Wackerbauer (1970) reported the use of cylindro-conical vessels for the production of German beers and claimed that the products were comparable in flavour to beers fermented in the traditional open tanks. Nathan (1930) claimed that by purging the beer with carbon dioxide at the end of the primary fermentation, the length of the lagering period could be subsequently reduced and in the U.S.A. this technique has gained widespread acceptance (Prechtl, 1961).

iv. Continuous fermentation - first attempts

As early as 1892 Delbruck proposed a system of continuous fermentation in which the wort was passed through a vessel where a high concentration of yeast was maintained within a porous cylinder. A fully continuous process utilizing a battery of stirred tanks was described by Van Rijn (1906). The tanks were placed on successively lower levels and the wort flowed from the top of the first tank to the bottom of the second tank and so on.

A simpler though not strictly a continuous process was that devised by Schalk (1906) consisting of a series of inter-connected tanks. The process was operated by seeding the first tank with twice the normal amount of yeast and after 18 - 48 hours half the contents of the first tank was fed into the second tank. Both tanks were then topped up with fresh wort and aerated. The fermentation was allowed to proceed to completion in the first tank but by a similar procedure the third tank was inoculated from the second tank. Material from the last tank was used to restart the first tank.

It is evident that even in the early stages of the development of continuous fermentation systems the principles employed differed widely. Many of the early systems suffered from insufficient safeguards against contamination by unwanted micro-organisms, whose presence was deleterious to the quality of the product. The flavour of beer is markedly affected by the concentration of minor constituents which may be present in quantities of a few parts per million or less, and it was because of the difficulties experienced in obtaining the desired flavour that many attempts to produce beer by continuous fermentation failed, and as a result interest in continuous systems lapsed. It was not until after World War II that any further development was undertaken in the Western World.

v. Modern developments in continuous fermentation

The types of continuous fermentation systems which have been proposed in the last 35 years have been very diverse. In order to compare the features of the various systems a method of classification is necessary.

In 1961 Herbert published an analysis of continuous culture methods according to their modes of functioning. All the known systems were divided into two main classes, which were called open

and closed. The open systems were described as those in which the micro-organisms flowed continuously from the fermenter in the effluent which represented a typical sample of the contents. The systems which yielded a cell free liquid were designated as closed. This classification has been represented graphically in Fig. O. Each major group was subdivided into homogenous and heterogeneous systems and each sub group into single stage and multi-stage systems. The systems which have been developed for beer production fall into an 'open' category.

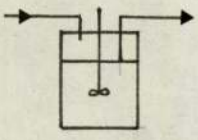
In the Soviet Union Malin (1940) published details of a system in which five vessels were connected in series. The wort flowed from the base to the head of the vessel. A pilot scale plant of this design (Markin, 1940) was operated and it was claimed to produce beer of an acceptable quality.

A continuous plant described by Wellhoener (1954)(Fig. 1.) employed a similar design to that of Markin (1940). The main fermentation tanks, however, were of unequal size and constructed as horizontal cylinders. Wort was fed into the first tank, of 4 kl capacity, from the top and passed on to the second and third fermentation tanks of 3 kl and 2 kl capacity respectively. Cooling elements were incorporated into the second and third tanks. The effluent beer was then passed to a series of three more tanks of 1.5 kl capacity, which were placed in the cellar and held at $0^{o}C$. In the first of these tanks the beer was clarified and passed to the top of the second tank where it fell through a layer of carbon dioxide, which served to wash the beer of the young flavour. The final tank served as a buffer vessel.

Experiments with a continuous system utilizing tanks of unequal size were undertaken by Czech workers (Hlavecek, Klazer and Khaler,

Fig. 0.

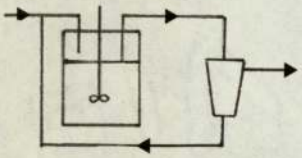
Types of Open Continuous Fermentation Systems (after Herbert, 1961).



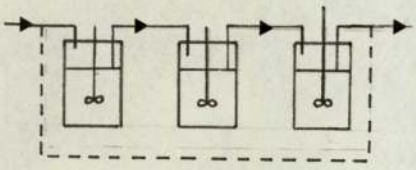
A. Homogeneous

1. Single stage

i. Stirred fermenter.

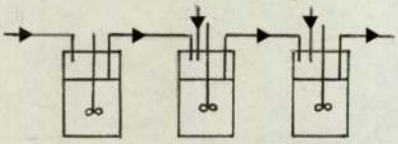


ii. Stirred fermenter with feed back.

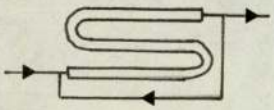


2. Multi stage

i. Simple stage



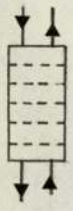
ii. Multiple substrate addition



B. Heterogeneous

1. Single phase

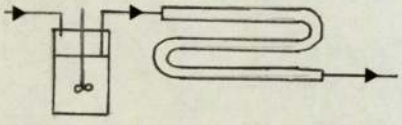
i. Pipe flow with feed back.



2. Multiphase packed towers.

i. Liquid - liquid

ii. Liquid - gas

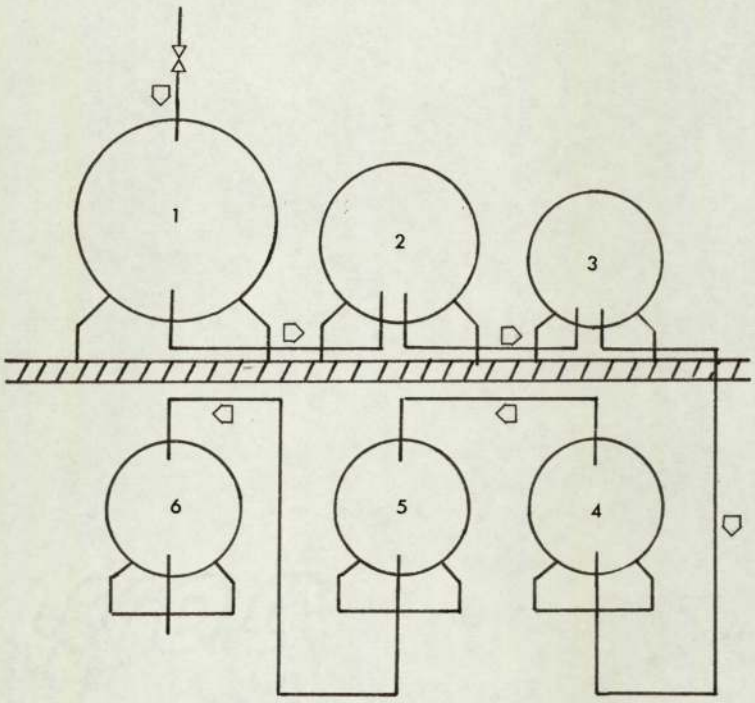


C. Mixed

i. Stirred tank feeding tubular reactor.

Fig. 1.

Continuous beer fermentation plant
(WELLHOENER 1954)



1 2 3 4 5 6 Fermentation vessels

1958, 1959). Using vertical cylinders they found that it was impossible to maintain the correct conditions, and that the quality of the product was inferior. A pilot plant using a two vessel system, in which the second tank was approximately twice the size of the first tank, succeeded in producing a beer with a similar analytical profile to that of batch produced beer. With this system the authors claimed a fully matured beer could be produced in 11 days.

In the Soviet Union a pilot plant incorporating a fermenter with an internal labyrinth has been constructed (Denshchikov, 1961b). The fermentation system consisted of five vertical tubular tanks connected in series. Secondary fermentation was carried out in conventional horizontal storage tanks and it was claimed that by operating at 22^{o} - 23^{o}C the plant was capable of producing beer within 4 days.

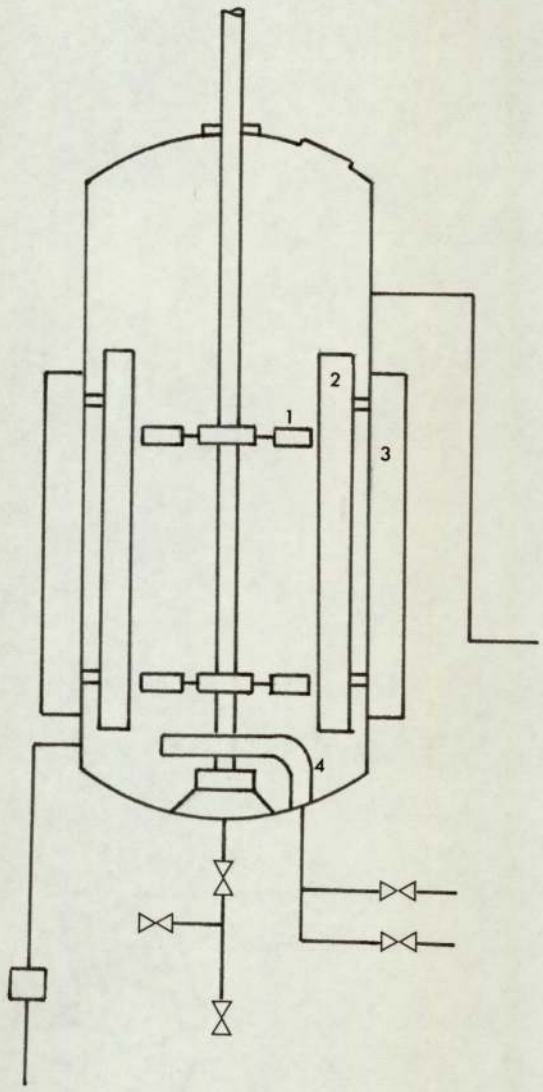
In many fermentation systems, mixing has been achieved by the use of mechanical agitators (impellers) such as in the Labatt's process (Geiger and Compton, 1957) or by arranging the fermenter to utilise the fermentation gas to produce circulation of the wort (Scholler, 1936). The use of mechanically agitated systems has become the normally accepted practice in the western world. The two stage system developed by J. Labatt of Canada (Geiger and Compton, 1957) consisted of three continuously stirred reactors in series. The fermenters were of similar construction to those used in the antibiotic industry (see Fig.2.) and incorporated two vaned disc impellers, baffles and a cooling jacket. Provision was made for injecting air or carbon dioxide below the lower impeller. The incoming wort was fed to the bottom of the tank and the effluent removed from the head.

In the first vessel aerobic conditions were maintained and the

Fig.2.

Stirred fermenter for continuous beer production.

(GEIGER and COMPTON 1957)



1 Impeller

2 Baffles

3 Attemperator jacket

4 Gas inlet

yeast concentration in the tanks of the second stage was controlled at approximately ten times the normal for batch fermentation by recycling part of the yeast from the effluent beer. By using a higher temperature (16°C) than is normal (5 - 8°C) for lager beer production, a rapid fermentation was achieved.

A similar system has been patented by Dominion Breweries Limited, New Zealand (Coutts, 1958). Up to four tanks could be employed but the usual process utilized a system of two tanks. Agitation was achieved by the use of a propeller type stirrer within a circulating cylinder.

A mixed system utilizing two stirred and two unstirred fermenters was developed by Ramsden and Son Limited in Britain (Williams, 1961). Closed tanks of increasing size were arranged on levels so that the fermenting wort cascaded from one tank to the next. Aerated wort was fed into the first stirred tank where yeast growth occurred, and then flowed to the second tank which was also stirred. The yeast which settled from the beer in the third and fourth tanks was recycled to the first. Conditioning occurred in the fourth tank and it was claimed mature beer could be produced in ten days.

More recently Bishop (1970) published details of the continuous stirred fermentation system employed by Watney Mann Limited in Britain. It consisted of two main vessels of equal size, the wort flowing into the base of the first tank and out of the head to the top of the second tank. A third tank with a conical base incorporating a foam breaking device and an internal cooling coil served as a yeast sedimentation vessel. The main fermentation tanks were not supplied with a gas sparger but the wort flowing into the first tank was oxygenated after sterilization.

In 1972, Harris and Watson published a description of a continuous unstirred fermentation process used by Scottish and Newcastle Breweries Limited. Two cylindro-conical vessels of 29 kl and 13 kl capacity respectively were connected in series. The first vessel was fitted with inverted cone baffles, which served to stabilize the yeast mass and maintain the concentration of yeast in the first fermenter between 5 - 8 times that in a normal batch fermentation. The fermented wort flowed into the second vessel which was chilled to facilitate yeast separation. The yeast was drawn off from the bottom of the second vessel and could be recycled. The authors claimed that it was possible to produce a fully fermented beer in approximately 12 hours.

Though the variations of stirred and unstirred systems of continuous fermentation described have been used in pilot plant trials or full scale production, many more designs of equipment have been tested on the laboratory scale.

A two stage stirred system was developed at the Brewing Industries Research Foundation (Hough and Rudin, 1958). These authors showed that in this system, which had no provision for recycle of yeast, there was no advantages to be gained by using more than two vessels, or by using vessels of unequal size. Beers of an acceptable flavour and similar analytical profile to that of conventionally produced beers could be obtained over a range of temperatures.

Another development of the stirred fermentation was apparent when Hough and Ricketts (1960) published details of the so-called V-tube fermenter. This equipment comprised a vertical tubular zone which was mechanically agitated and an inclined tube which joined the vertical vesel at the base. The unagitated zone within the sloping tube provided an area in which flocculent yeasts could settle from the beer and return to the main fermentation zone, thus allowing a

very high concentration of yeast to be attained. With a suitable strain of yeast rapid fermentations were possible.

Denshchikov (1961a) described a tubular laboratory fermenter 3.5 cm in diameter and 29.3 m. long, but operational problems were encountered and the unsuitability to scaling up outweighed any advantages to be gained with the system. Portno (1967) worked with a similar fermenter and found that premixing of the yeast and wort allowed conditions of true heterogeneity to be attained and as a result the product was almost identical to a batch produced beer.

A development of the continuously stirred multivessel system was described by Portno (1969). The fermenter was arranged as a vertical tube which was separated into 5 chambers by discs attached to the stirrer shaft. It was possible to operate this fermenter at a dilution rate of 1.0, though some signs of instability were apparent at this rate.

The adoption of continuous processes for mashing and fermentation has been a result of the realisation that production economics of the continuous system can be more favourable than that of the batch system. Often quoted savings for continuous systems are the following:

i. Reduced labour costs.

ii. Minimisation of cleaning.

iii. Stability of product quality.

iv. Savings in space.

Against these advantages there are a number of complications which the adoption of continuous fermentation systems introduce. Firstly, it is essential that contamination of the system with "foreign" micro-organisms is avoided because the quality of the beer will most surely be affected adversely. It is also true that

continuous systems of fermentation are less flexible than batch methods of beer production because changeover of a continuous system from one beer to another may necessitate stopping the system, cleaning out and re-starting, which can be a lengthy procedure.

Thorne (1968) has expressed some fears that the quality of the yeast mass may alter during prolonged operation of continuous fermentation and that the use of elevated temperatures to produce rapid fermentation may adversely affect the beer quality.

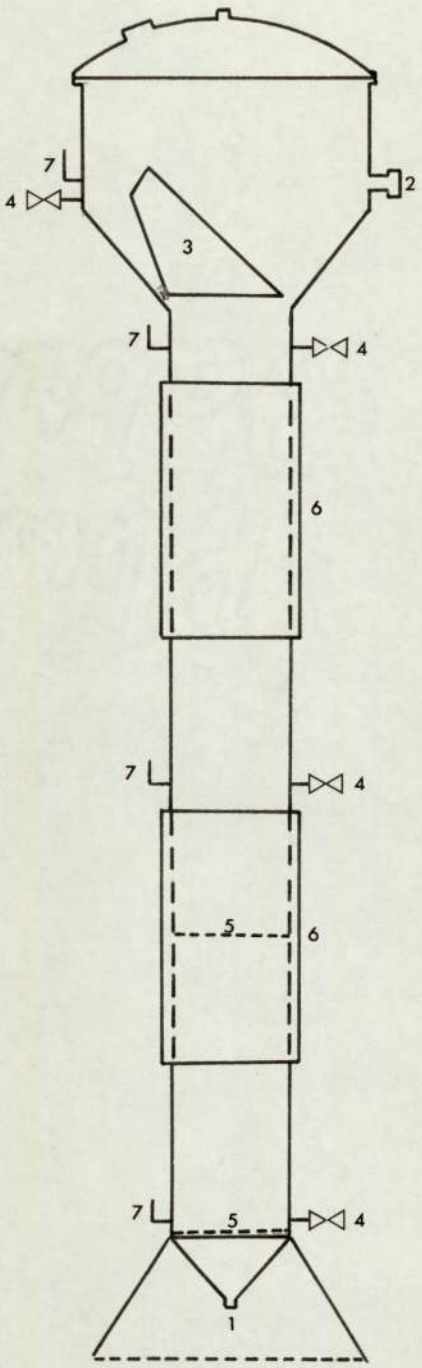
vi. The Tower Fermenter

A continuous fermentation system in the form of a tower was described by Victerero (1948) but the modern tower fermentation system was patented for the A.P.V. Co. Ltd., by Watson and Shore (1963). The fermenter takes the form of a vertical tube with an extended section at the head (Fig. 3.). Besides the physical shape the main difference between the tower fermenter and most other commercial processes of continuous fermentation is that the tower is operated as a single stage system. The wort is fed into the base of the fermenter and passed through the yeast mass, the elongated form of the fermenter allowing a range of conditions to exist within the fermenter (Royston, 1966). The fully fermented beer overflows at the head of the fermenter through a device which separates the yeast from the beer.

In order to reduce gas channelling and prevent back mixing of the yeast and beer it was found necessary to incorporate baffles within the tubular section of the fermenter (Klopper, Roberts, Royston and Ault, 1965). Both flocculent and non-flocculent yeasts were examined in the tower fermenter by these workers but it was found that non-flocculent yeasts tended to be washed out of the fermenter at low rates of production. When using specially selected strains of

Fig.3.

Schematic diagram of the APV tower fermenter



1 Wort inlet 2 Beer outlet 3 Clarifying tube

4 Sample ports 5 Baffle 6 Attemperator jacket

7 Temperature indicator

Saccharomyces cerevisiae it was possible to achieve fermentation of ale worts in 4 hours which represents approximately a 20 fold reduction on the batch fermentation time. When using unaerated wort the beer produced had an acceptable but distinct flavour.

In the early work with the tower fermenter, restricting the yeast growth was considered advantageous because more efficient conversion of carbohydrate to alcohol was achieved (Royston, 1966). The reduced growth of yeast led to the production of beers with high levels of nitrogen, a problem which was overcome by the use of low nitrogen worts. Further work suggested that by aerating the wort and allowing a greater degree of yeast growth, beers with normal nitrogen contents could be produced (Rainbow, 1970). An additional advantage was that the distinct estery flavour associated with tower beer disappeared.

Process Considerations

In the previous section the variety of continuous fermentation processes has been illustrated, but all the different techniques are governed primarily by the need to produce an acceptable product. To this end a great deal of research has been conducted to establish the factors which have a major influence upon the performance of continuous fermentation.

To simplify the discussion of these factors they are best dealt with separately though by the complex nature of the process a small alteration of one variable may have profound effects upon the fermentation and the quality of the beer.

i. Oxygen

In the batch production of beer the early stages of fermentation are characterized by the presence of oxygen in the wort. During the first 5 - 10 hours of a fermentation there is no appreciable increase

in cell numbers and it is this period which has been termed as the lag phase. The length of the lag phase depends largely upon the physiological condition of the yeast but it is shortened if the wort is short of oxygen.

The role of oxygen in brewery fermentation appears to be that of a growth factor. In a wort containing 10 ppm O_2 the quantity of carbohydrate which could be utilised by the hexose monophosphate and tricarboxylic acid pathways is less than 0.01% of the total carbohydrate. Therefore, the major role of the oxygen may be to induce enzymes (Lie, 1965). In laboratory experiments to determine the effect of oxygen Markham (1969) showed that wort oxygen concentrations above 8 ppm had little effect upon yeast yield.

The effect of aeration upon continuous fermentation systems of the type of Hough and Rudin (1958) and Hough and Ricketts (1960) have been reviewed by Rainbow (1970). The findings of the various workers may be summarized as:

a. Low aeration rates increase the beer production rate.

b. Oxygen is the growth limiting factor.

c. At high aeration rates the Pasteur effect is marked and the rate of ester production is reduced.

The only publication dealing with the effects of aeration in tower fermenters is that of Ault et alia (1969). The yeast growth rate was increased by aeration and the levels of esters in the beer was reduced from the abnormally high levels which were found when the tower was operated anaerobically. In addition yeast viability was improved.

ii. Temperature

The temperature is an important factor which influences the rate of yeast growth and fermentation. In ale fermentations the temperature range is $15^{o}C$ - $25^{o}C$ and within this range the growth and fermentation coefficients show an almost linear increase (White and

Munns, 1951). Maule (1972) has pointed out that the production rate of a continuous chemostat type beer fermenter may be controlled by suitable adjustment of the temperature and dilution rate without affecting the yeast concentration within the fermenter.

In the operation of continuous systems of fermentation it has become an accepted practice to conduct the fermentation at a temperature which is higher than that normally attained in a batch fermentation. Makinen (1971) reported that for continuous lager production a temperature of 15°C was optimal. Below 10°C the rate of beer production was so slow that the advantages of continuous fermentation were nullified.

Merritt (1966) studied the influence of temperature upon the progress of batch fermentations with Saccharomyces cerevisiae and found that maltase activity and alcohol production was maximal at 25°C. At 30°C the yeast yield and the production of glycerol and higher alcohols reached a maximum.

The use of elevated temperatures in lager fermentations has been reported to increase the production of vicinal diketones (Voerkelius, 1961).

The evidence in the literature indicates that the use of elevated temperatures will accelerate fermentation but may result in a beer with an abnormal flavour so that it may be necessary to sacrifice an increase in production rate to obtain an acceptable product.

iii. Flocculence

Flocculation is a phenomena of vital importance to the brewer. The term flocculation is used to describe a process which can be conveniently regarded in two stages:-

a) Aggregation of the yeast cells into clumps.

b) Separation of the yeast clumps from the liquid in which it is suspended.

In the batchwise brewing of ale when the yeast flocculates it rises to the surface of the beer and forms a 'head' which may be skimmed off at intervals during the fermentation. Yeasts which are highly flocculative separate early in the fermentation and so produce beers which are less fully fermented and contain more residual sugar. Less flocculent or powdery yeasts produce well fermented beers but clarification of the beer may be slow. Subsequent autolysis of the yeast may produce undesirable flavours.

The practice of lager brewing is also governed by the considerations mentioned above except that Saccharomyces uvarum does not form a yeasty head during fermentation but settles to the bottom of the vessel. This difference in behaviour led to the use of the terms 'top yeast' to describe Saccharomyces cerevisiae and 'bottom yeast' for Saccharomyces uvarum. Some modern techniques have made this difference in behaviour less distinct since it is known that ale yeasts will settle to the bottom of a fermentation vessel under some conditions.

The modern continuous fermentation techniques require a yeast with flocculation characteristics which are specially suited to the system in which it is to be employed. The use of non-flocculent yeast is favoured for the production of beer in systems employing the chemostat principle since the use of a highly flocculent yeast can prevent the attainment of steady state conditions (Rudin and Hough, 1959). When a mixed culture is used in a chemostat type fermenter, the non-flocculent yeast will eventually predominate (Makinen, 1971).

The two heterogeneous fermentation systems in use in Britain, the A.P.V. tower fermenter (Shore and Watson, 1963) and the Scottish and Newcastle Breweries system (Harris and Watson, 1972) require the use of a highly flocculent yeast.

The characteristics of the A.P.V. tower fermenter have been

reported previously by Klopper et alia, (1965) and Royston (1966).

The system described by Harris and Watson (1972) differs from the A.P.V. tower fermenter in that it is a two stage system, but the success of the method depends upon the maintenance of a high yeast concentration in the fermenters, a situation which can only be achieved by employing a highly flocculent yeast.

The subject of flocculation has been reviewed by Comrie (1952), Jansen (1958) and Rainbow (1970), and further discussion is not warranted in this context.

iv. The wort

Brewers wort is a most complex medium containing fermentable and non-fermentable carbohydrates, amino acids and peptides, inorganic trace elements, growth factors and other complex organic molecules. The wort used by brewers has been developed for the most satisfactory production of beer by batch fermentation and therefore it is not suprising to read in the literature that the use of a normal (batch) wort for continuous fermentation may present some problems.

Coutts (1966) reported that the quality of beer produced from a continuous system was improved by storing the wort at $0^{o}C$ before use. In the early work upon the tower fermenter it was found that the beers contained high levels of nitrogen but this problem was overcome by the use of low nitrogen worts (Rainbow, 1970). There is also evidence to suggest that removal of suspended solids from the wort before fermentation is beneficial to the quality of the beer (Malin, 1940). In 1971 Makinen reported the results of research upon continuous lager fermentation and came to the conclusion that to obtain a beer of normal quality it was necessary to tailor the wort to the fermentation process employed.

By examining the literature it is apparent that the quality of

beer is influenced by the interaction of many factors and that a quantitatively minor alteration of any of these may have a profound effect upon the quality of the product.

viii. The development of a laboratory scale tower fermenter

The successful operation of a tower fermenter depends to a large degree upon the selection of a suitable yeast. Those strains which are suitable for batch fermentations are not necessarily those which will give acceptable results in the tower fermenter.

When tower fermenters were first used commercially only one suitable strain of flocculent yeast was known. Consequently a screening programme was undertaken by the University of Aston on behalf of the A.P.V. Company Limited. A laboratory tower fermenter was constructed in glass to a design of D'Eath and Greenshields, (1967) (Fig. 4).

A number of yeasts were obtained to establish a continuous fermentation culture collection (C.F.C.C.). Initial screening of the yeasts was undertaken to select flocculent types, by use of a sedimentation test (Helm, Nøhr and Thorne, 1953). When operating with the selected yeasts in the tower fermenter it became apparent that certain yeasts were more flocculent than others, though the sedimentation test had not distinguished between flocculent strains. Accordingly, a modified sedimentation test was developed and it proved possible to gain a more accurate assessment of flocculence (Greenshields et alia, 1972).

Investigation of the yeasts held in the C.F.C.C. showed that approximately half may be suitable for use in the tower fermenter and that the ale type yeasts could be classified as below, according to their physical and fermentation characteristics (Greenshields and Smith, 1971).

Fig. 4

A diagramatic representation of the Laboratory Tower Fermenter

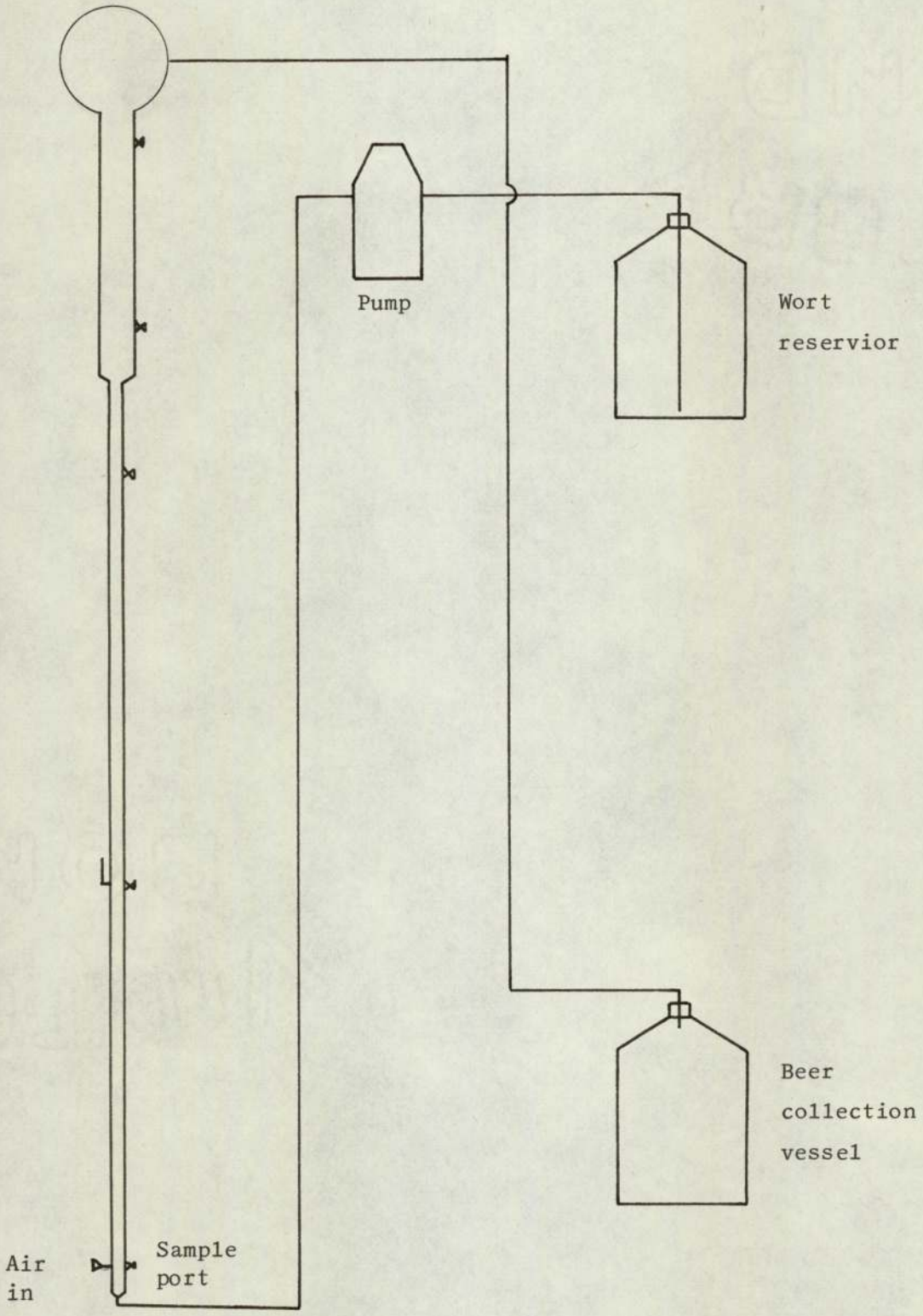
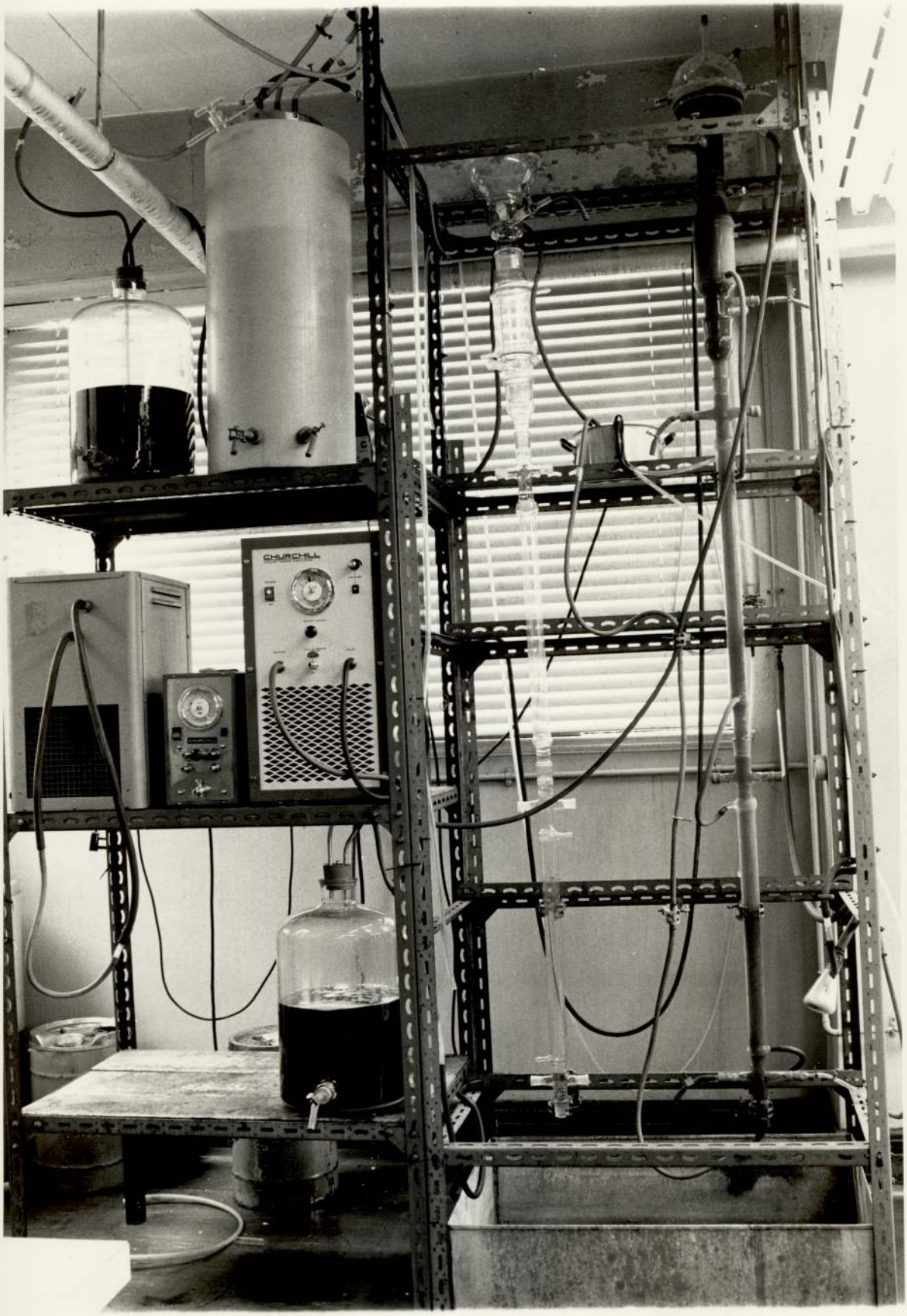


Plate 1.

The laboratory tower fermenter and ancillary equipment.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i. Non-flocculent - the yeast did not attain high concentrations in the fermenter and was easily washed out.

ii. Flocculent - physically limited - these yeasts attained a high concentration but above a critical wort flow rate the yeast washed out of the fermenter.

iii. Flocculent - fermentation limited - these yeasts attained high concentrations but at a critical flow rate, full fermentation of the wort was no longer achieved.

The work presented in the first section of this thesis is a continuation of the work reported by Greenshields and Smith (1971) and describes the author's experience with three strains of yeast.

Subsequent sections deal with the results of investigations into yeast genetic stability, the continuous fermentation of lager and, finally, molasses.

SECTION I

ASSESSMENT OF YEAST IN THE TOWER FERMENTER

i. CFCC 26

ii. CFCC 52

iii. CFCC 54

INTRODUCTION

In this section the results of investigations upon three strains of yeast, one each of the species Saccharomyces cerevisiae, Saccharomyces uvarum, and Saccharomyces diastaticus have been reported. The procedure followed during the investigation of the yeasts was developed in the Fermentation Technology Laboratory at the University of Aston in order to select yeasts which may be suited to the Tower fermenter.

The yeasts were primarily selected by means of a simple flocculation - sedimentation test (Greenshields et alia, 1972) and subsequently cultivated in laboratory scale tower fermenters in order to assess their performance and behaviour under the conditions of continuous tower fermentation.

A. MATERIALS AND METHODS

i. The Tower fermenter

The laboratory tower fermenter (see Plate 1) was of all glass construction and was supplied by Harmon Apparatus, Harbourne, Birmingham. The design, based upon a commercial tower fermenter, was that of D'Eath and Greenshields (1967). The overall height of the fermenter was 255 cm and the depth of liquid within the fermenter 235 cm. The tubular section consisted of a 175 cm length of 2.54 cm diameter and a 54 cm length of 8 cm diameter. The expanded head section was spherical and contained liquids to a depth of 6 cm. Sample ports were provided at 15 cm, 80 cm, 160 cm, 185 cm and 235 cm, above the point of wort entry.

Part of the length of the tubular section was jacketed to allow circulation of attemperated water.

ii. Ancillary equipment

A 40 litre heavy duty polypropylene container served as a reservoir for the wort and a 20 litre glass aspirator was attached to the fermenter outlet to collect the beer. A thermocirculator (Churchill Instrument Company) was used to provide attemperated water for the jacketed section of the tower.

A flow inducer (Watson Marlow) served as a wort pump.

iii. The wort - Malt Extract Syrup

Unhopped malt syrup batch number CME 1284 was supplied by E.D.M.E. Limited, Mistley, Essex.

iv. The yeast cultures

Details of the yeast cultures are listed below:

CFCC 26D - *Saccharomyces uvarum* ex bottle Bass Red

CFCC 52 - *Saccharomyces uvarum* AJ.2155 ex A. Jorgensen A/S

CFCC 54 - *Saccharomyces diastaticus* G.606 ex A. Guinness Ltd.

v. Routine analytical procedure

The following parameters were measured routinely:

a. Wort original gravity.

b. Beer present gravity.

c. pH.

d. Total acidity (Titration against N/10 NaOH).

In addition checks for contaminating micro-organisms were undertaken by microscopy.

vi. Preparation of the wort

The malt wort of specific gravity 1.050 was prepared by adding a suitable quantity of water to the malt syrup. This wort was recirculated through pasteurisation apparatus at 98^{o} - $100^{o}C$ for two hours and then pumped into the wort receiver which had been steamed out for two hours.

vii. Preparation of the tower fermenter

Before use the tower fermenter was thoroughly washed. Sterilisation was achieved by free steaming for 20 - 24 hours. All connections to the wort and beer tanks were made by rubber tubing which had been sterilised by autoclaving at 1.05 kg/cm^2 for 15 minutes.

viii. Preparation of the yeast culture

The culture for inoculation was prepared from a master culture by propagation in malt wort of specific gravity 1.050. Approximately 1000 ml of actively fermenting wort was used to inoculate the tower fermenter and then the tower filled with fresh wort. After 18 - 24 hours the pump feed from the wort reservoir was switched on and a continuous flow maintained at approximately 125 ml/hr at first, until the beer flowing from the head of the tower reached a specific gravity of 1.010. The flow rate was subsequently adjusted to obtain

the maximum consistent with production of beer with a specific gravity 1.010. Throughout the period of yeast propagation aeration was achieved by introducing sterile air at the base of the tower fermenter.

ix. Normal operational procedure

Anaerobic conditions were established in the tower fermenter when the yeast had grown sufficiently to fill the fermenter. A period of 48 to 72 hours was allowed from the time the air supply was stopped before any analyses were made, to allow the yeast mass to adapt to the new conditions.

The wort flow rate was then increased daily by increments of approximately 1.5 litres/day until physical washout of the yeast or some limitation of fermentation properties was observed. Fermentation limitation is considered to have occurred when the apparent attenuation rises above 80 % (Greenshields and Smith, 1971).

x. Expression of the production rate of the fermenter

The rates of beer production have been expressed as a Volumetric Efficiency (E) which is calculated as:

$$E = \frac{v}{V} = \frac{\text{Volume of beer produced in 24 hours}}{\text{Void volume of fermenter}}$$

when using wort of specific gravity 1.050 to produce beer of 80 % attenuation. This figure is not accurate as an indication of fermentation rate because it ignores the fact that the void volume of the fermenter is considerably less than the physical volume due to the presence of gas and yeast within the fermenter. However, this figure does indicate the actual volume of beer produced.

The subscript on E is an indication of the original gravity of the wort.

For example, the subscript E_{50} is arrived at by the calculation below:

E_x where x = (O.G. - 1.000) x 1000

x = (1.050 - 1.000) x 1000

x = 50

xi. Superficial Liquid velocity (S.L.V.)

Superficial liquid velocity is calculated as:

$$\frac{\text{Volume per unit time}}{\text{Cross-sectional area of fermenter}} = \text{S.L.V.}$$

the units of S.L.V. used are cm/sec.

All the values quoted in this section refer to the S.L.V. in the 2.54 cm diameter section of the fermenter.

xii. Yeast concentration

The yeast concentration in the fermenter was determined at the termination of the experiment. The total contents of the fermenter were transferred into a container and thoroughly mixed. 100 ml samples were removed and centrifuged for 5 min at 5000 r.p.m. in an M.S.E. bench centrifuge. The supernatant was decanted and the yeast weighed. This weight has been reported as the centrifuged wet weight. The dry weight of the sample was measured after drying in an oven at 105°C for 24 hours.

B. RESULTS

i. Yeast CFCC 26D

The yeast, a strain of Saccharomyces uvarum was isolated from yeast CFCC 26. This isolation gave a Modified Burns No. 82 when tested by the method of Sharp (Greenshields et. al. 1972).

The fermenter was operated at 20°C according to the standard procedure (page 29), to establish the limiting Volumetric Efficiency (E_{50}) with a wort original gravity 1.050.

i.a. Gross Morphology

In the fermenter this yeast aggregated to form very large flocs up to 0.5 cm diameter. The individual flocs also showed a tendency to coalesce and form solid plugs which occupied the full diameter of the tower. These plugs were fairly stable structures and showed little tendency to break up at low wort flow rates. As a result pockets of fermentation gas liquid were trapped beneath the plugs which were forced up the tower.

Some of the large flocs were observed to float to the head of the tower, where on occasions they blocked the beer effluent pipe, and caused the liquid level in the fermenter to vary.

i.b. Limiting Flow Rate

The exceptionally flocculent nature of this yeast limited the maximum rate at which the fermenter could be operated, because above a volumetric E_{50} = 1.6 the yeast was forced up the fermenter by the liquid. This rate corresponded to S.L.V. 1.17 x 10^{-2}cm/sec in the 2.54 cm section.

i.c. Analysis of the beer

The analysis of the beer produced at the limiting flow rate is presented below:

Wort O.G.	1.0495
Beer P.G.	1.0095
pH	3.0
Total acidity (as acetic acid)	0.19%w/v

i.e. Fermentation gradient in the fermenter

The fermentation gradient within the fermenter was established at a volumetric efficiency of 1.6. The results have been recorded in Graph 1.

i.f. Comment

It has been demonstrated that this yeast is of a type which falls into the category of physically limited yeast in the classification of Greenshields and Smith (1971).

Analysis of the beer indicated that fermentation of the wort was satisfactory at wort flow rates up to the limiting flow rate.

The fermentation gradient was of the same pattern as that observed in a commercial fermenter under stable conditions (Ault et alia, 1969). From the information obtained with this yeast it seems reasonable to assume that the limiting rate was governed solely by the morphology of the yeast in the fermenter. If the solid plugs of yeast had not formed then it is possible that a higher rate of operation could have been reached whilst still achieving a satisfactory fermentation.

ii. Yeast CFCC 52

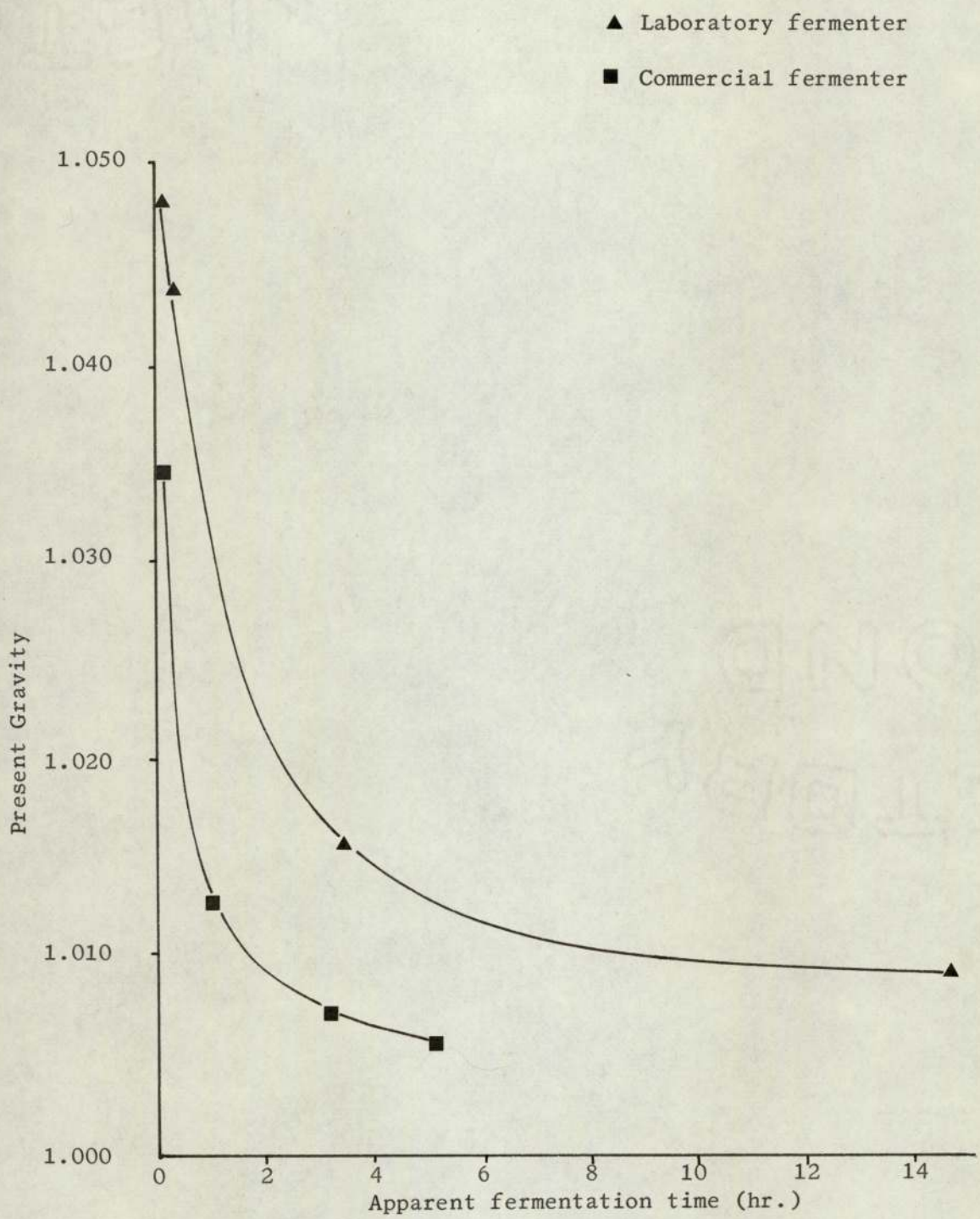
This yeast was a strain of Saccharomyces uvarum. The flocculence test gave a Modified Burns No. 77.

ii.a. Gross Morphology

In the tower fermenter this yeast formed small flocs up to 1 mm

GRAPH 1.

The change in present gravity of the wort with time in a commercial and the laboratory tower fermenter



The data for the commercial fermenter have been taken from the publication of Ault et alia (1969).

diameter and was well dispersed throughout the fermenter. Occasional foaming was noted but no specific reason for this was ascertained.

ii.b. Limiting flow rate

At volumetric efficiencies (E_{50}) greater than 1.4 the present gravity of the beer rose sharply.

Washout of the yeast occurred at a volumetric efficiency (E_{50}) of 2.0 which corresponded to a S.L.V. of 1.07×10^{-2} cm/sec in the 2.54 cm section.

ii.c. Analysis of the beer

At volumetric efficiencies of 1.4 the beer produced gave the following analysis:

Wort O.G.	1.0495
Beer P.G.	1.0175
pH	3.9
Total acidity (as acetic acid)	0.23 g% w/v

At washout a beer with the following analysis was produced:

Wort O.G.	1.0495
Beer P.G.	1.020
pH	3.9
Total acidity (as acetic acid)	0.23 g% w/v

ii.d. Comment

Throughout the experiment this yeast failed to produce a beer with a specific gravity lower than 1.016 from a wort of original gravity of 1.0495. The malt extract was part of the same batch as that used for the experiment upon yeast CFCC 26D which gave a beer of present gravity 1.0095.

The reason for this discrepancy was not established, but it will be shown later that yeast CFCC 52 was capable of producing a

satisfactory beer from a different malt syrup. Therefore it appears that the wort may have been unsuitable in some respect.

iii. Yeast CFCC 54

This yeast, a strain of Saccharomyces diastaticus, gave a Modified Burns No. 153 by Sharp's Test.

iii.a. Morphology

In the tower fermenter the yeast formed a bed consisting of pellets up to 0.5 cm diameter. As the wort flow approached the limiting rate some axial mixing was noted and the yeast bed began to expand.

iii.b. Limiting flow rate

Substantial washout of the yeast occurred at volumetric efficiency (E_{50}) 3.0 when the S.L.V. in the 2.54 cm section was 2.21 cm/sec.

iii.c. Analysis of the beer

The fermentation of the wort was satisfactory at all flow rates up to that at which washout occurred. Analysis of the beer produced at the limiting rate is given below:

Wort O.G.	1.051
Beer P.G.	1.008
pH	4.6
Total acidity (as acetic acid)	0.24 g% w/v

iii.d. Yeast concentration

The average yeast concentration reached 35 g/100 ml measured as centrifuged wet weight (7.95 g/100 ml dry weight).

iv. Additional investigations upon CFCC 54

I am indebted to Mrs. J. Davies for her assistance in operating the fermenter and performing analyses during the course of the work presented below.

The tower was operated using worts of various specific gravities between 1.042 and 1.092. In all other respects the procedure described under 'Methods and Materials' was followed.

iv.a. Yeast washout related to wort original gravity

The flow rates at which washout occurred were established for wort of various specific gravities. The results have been presented in Graph 2.

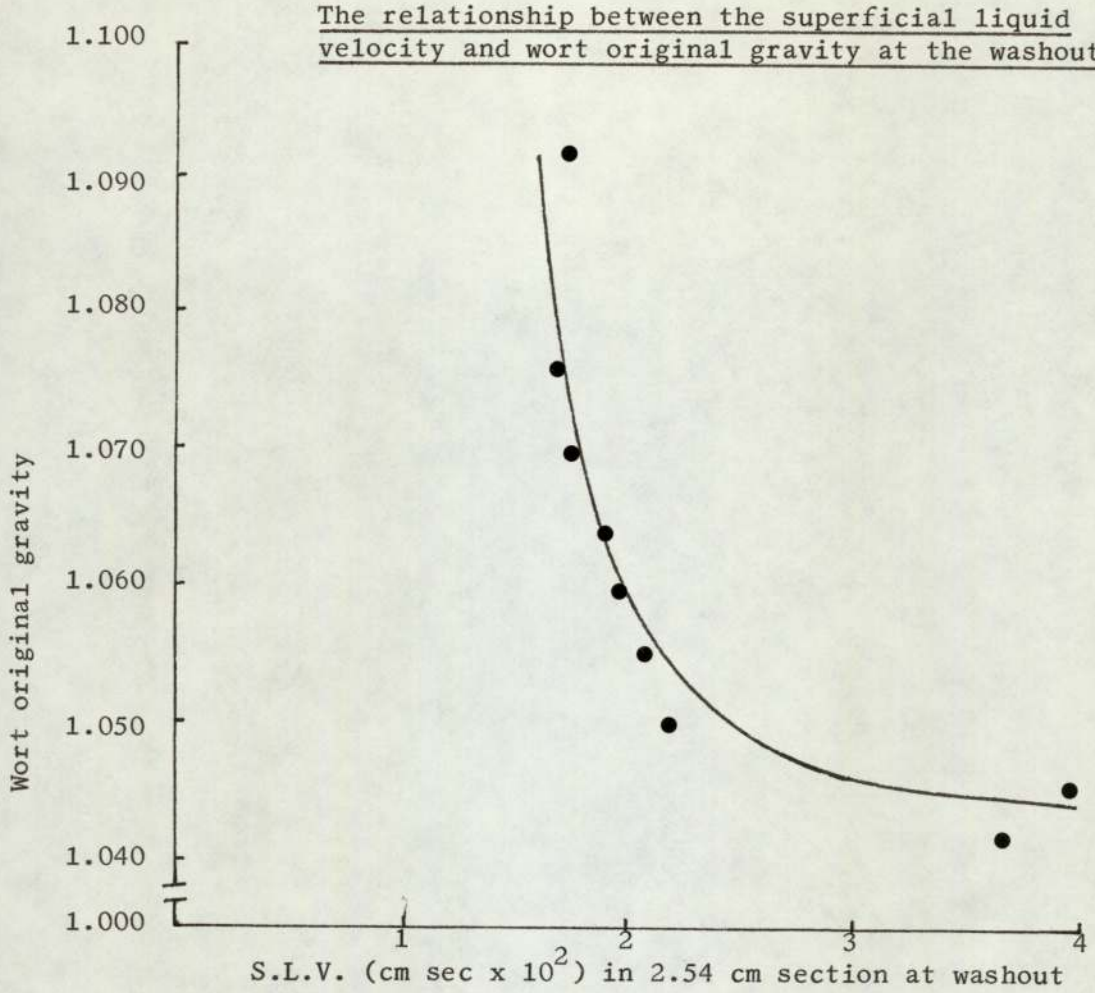
Instability of the yeast mass was apparent at relatively low flow rates when worts of specific gravity greater than 1.050 were fermented. When low gravity worts were used the limiting flow rates were considerably higher. This type of relationship has been reported previously for the yeast NCYC 1026 (Greenshields and Smith, 1971).

iv.b. Fermentation performance related to wort original gravity

By examination of the results for specific gravity of the beer produced at the washout flow rate, it was evident that the high gravity worts were not completely fermented. When the results were plotted graphically (Graph 3) it was apparent that worts of an original gravity greater than 1.065 were incompletely fermented.

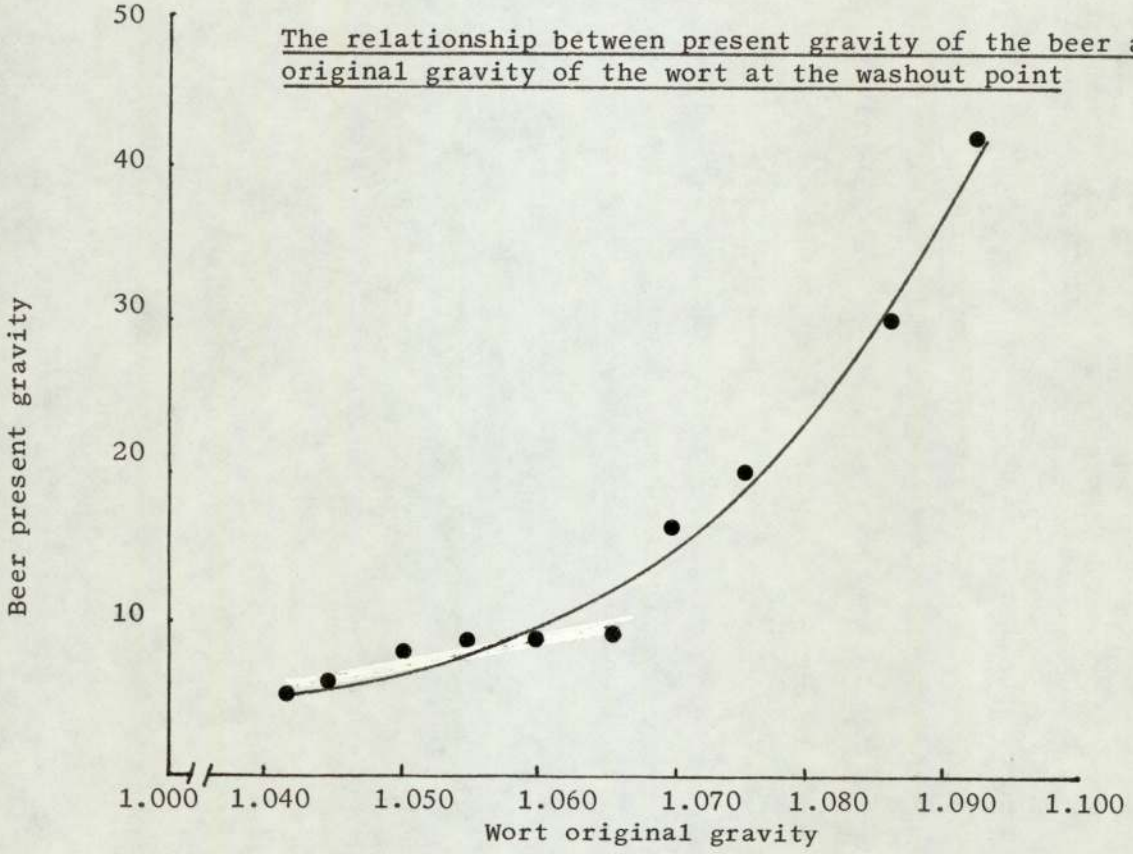
A further analysis of the results indicated that though the fermentation of high gravity worts was incomplete, the maximum drop in specific gravity occurred when worts of original gravity 1.075 were fermented (Graph 4).

The relationship between the superficial liquid velocity and wort original gravity at the washout point



GRAPH 2.

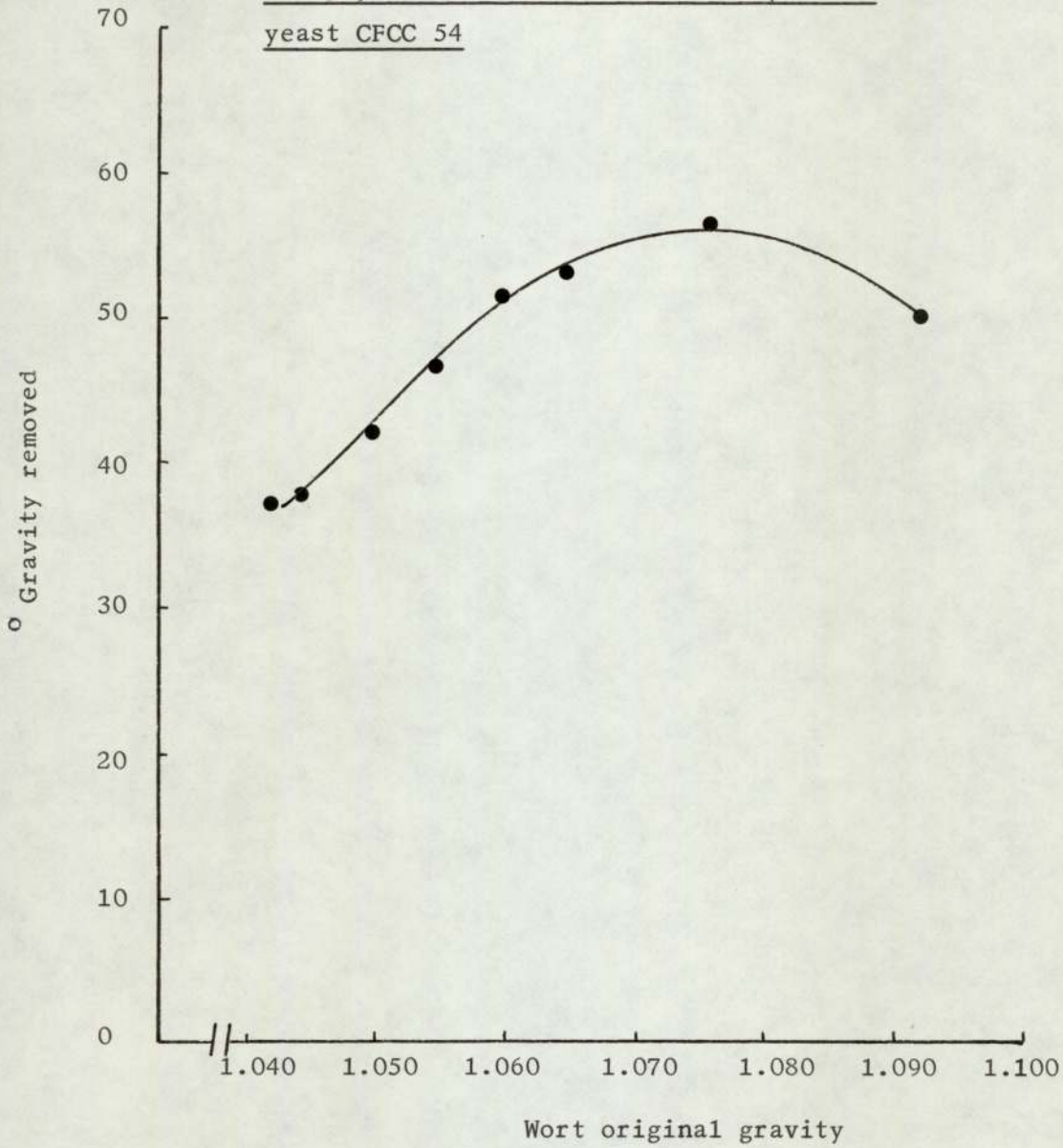
The relationship between present gravity of the beer and original gravity of the wort at the washout point



GRAPH 3.

Graph 4

The relationship between ^{o} gravity removed and wort original gravity when operating at the physical limitation efficiency with yeast CFCC 54



DISCUSSION

The concentration of yeast, and the optimum flow rate of the wort through a tower fermenter are governed to a large extent by the morphology of the yeast. The prevailing morphology is the result of interaction of two factors - the agitation due to the upward flow of liquid and gas, and the degree of flocculence of the yeast. The latter is a function of yeast strain, but factors such as wort concentration and the metabolic condition of the cells have an influence. The agitation in a tower fermenter is slight compared to that in other systems such as a continuously stirred tank reactor. Therefore the yeast is able to aggregate into fairly large stable flocs which can settle against the stream of upflowing liquid.

In the case of yeast CFCC 54 the flocs which formed were sufficiently dense to settle to the base of the fermenter. Subsequently the flocs coalesced to form a solid plug of yeast which was relatively impervious to both the gas and liquid. In consequence, at a critical flow rate, the yeast was forced up the fermenter and washout occurred.

In the case of the less flocculent yeast, CFCC 52, the agitation within the fermenter was sufficient to ensure the yeast remained dispersed. As a result when the velocity of the upflowing liquid reached a certain critical value the flocs were no longer able to settle and were carried out of the fermenter.

These two examples serve to illustrate the extremes of physical behaviour which are seen in the tower fermenter. It is not difficult to deduce that a yeast which forms flocs of an intermediate size should be most suitable for use in this system.

It is therefore not suprising that of the three yeasts investigated the one which performed satisfactorily showed an intermediate morphology. This yeast, CFCC 54 was capable of remaining

in the fermenter at wort flow rates up to almost double that at which yeast CFCC 26D washed out.

The very flocculent behaviour of yeast CFCC 26D could present another problem. It has been calculated by Greenshields and Smith (1971) that pore diffusion is the factor controlling the rate of fermentation in flocs greater than 0.1 cm diameter. Therefore it seems likely that cells at the centre of large flocs will be subject to poor nutritional conditions and hence yeast death and autolysis is a possibility which cannot be ignored, since the effect upon beer quality could be serious.

The present gravity of the beer produced by yeast CFCC 26D was within narrow limits (1.009 -1.0095) at all wort flow rates up to that at which washout occurred. This finding is in agreement with that of Ault et alia (1969) who reported that under stable conditions the tower fermenter can only produce a fully fermented beer.

The present gravity of the beer produced by CFCC 54 was consistently lower than that of the beer from yeast CFCC 26D. This phenomenon is known as 'super attenuation' and is due to the ability of CFCC 54 to utilize higher maltosaccharides such as maltotetraose, maltopentaose and maltoheptaose. The beer produced by CFCC 54 (G. 606) lacks 'body' and may contain an undesirable high level of ethyl acetate (Gilliland, 1971).

The ability of strains of Saccharomyces diastaticus to utilise higher maltosaccharides has been exploited by vinegar brewers, who for economic reasons require the maximum yield of alcohol from a malt wort (Greenshields and Smith, 1974). It seems that the yeast CFCC 54 could be ideally employed in the continuous fermentation of vinegar charging wort.

When fermenting worts of original gravity less than 1.065, CFCC 54 behaves as a flocculent - physically limited yeast after the classification of Greenshields and Smith (1971). However, when worts with an original gravity greater than 1.065 are used the performance of the fermenter is limited by the fermentation rate of the yeast.

The work that has been presented in this section illustrates the usefulness of the laboratory scale fermenter in the investigation of tower fermenter processes. By using this small scale system it has been possible to select a number of yeasts rapidly. The results presented in this section form part of an extensive screening programme during the course of which more than fifty yeasts have been studied.

SECTION 2

ASSESSMENT OF GENETIC STABILITY OF YEAST IN CONTINUOUS FERMENTATION

i) CFCC 1

ii) CFCC 70

INTRODUCTION

The success of tower fermentation depends upon the selection of a yeast with suitable brewing and flocculence characteristics. It is obvious that once a satisfactory yeast has been discovered, the stability of the strain is of prime importance.

However, there have been reports (Brown and Hough, 1965; Watson and Hough, 1966) of changes in the morphological characteristics of yeast during continuous fermentation. Spontaneous genetic mutations in yeast were first stated by Winge (1944) to be a common phenomenon, and since then numerous alterations in the morphological and biochemical characteristics have been uncovered.

Thorne (1951) showed that flocculence was a genetically controlled and that flocculence was dominant to non-flocculence. Furthermore the mutation rate from flocculence to non-flocculence was high. However, Gilliland (1951) considered that there was no clear evidence whether flocculence or non-flocculence was dominant.

In the light of the known genetic instability of yeast, Thorne (1968a) questioned the wisdom of using a continuous fermentation for the production of beer. In Thorne's opinion the use of stirred fermentation systems for the continuous production of beer was almost certain to favour the proliferation of mutants and the results of studies (Thorne, 1968b; Thorne, 1970) upon two strains of Saccharomyces uvarum confirmed this belief.

The first report (Thorne, 1968b) indicated that after nine months continuous operation approximately half the cells had mutated. The mutations included loss of flocculence, reduction of fermentation efficiency, reduction of final attenuation, change of growth rate and production of undesirable beer flavours. One mutant even appeared to have lost its claim to membership of the parental species.

Investigation of a second strain of Saccharomyces uvarum also showed that numerous mutations had occurred, though the pattern of mutations was different from that observed with the first strain (Thorne, 1970).

These findings indicated that the possibility of substantial changes in the yeast when operated for long periods in a continuous fermenter cannot be ignored. For the tower fermenter in particular a loss of flocculence by the yeast would be disastrous, though some of the other mutations could prove detrimental to beer quality. Less problematic would be the presence of yeast mutants of reduced fermentation efficiency or growth rates since the high yeast concentrations would probably mask any adverse affects the presence of such mutant strains might have. However, Thorne (1970) has shown that the undoubtedly more serious mutation, leading to increased production of diacetyl, can be associated with reduced growth rate and fermentation efficiency.

It was decided to investigate whether mutation of the yeast would present practical problems in the tower fermentation of beer. To this end the laboratory fermenter was operated continuously for four months. In addition a quantity of yeast of the same strain was removed from a commercial tower fermenter which had been in continuous use for one year.

A. MATERIALS AND METHODS

i. The Fermenter and Operation conditions

The tower fermenter, similar to that described in the previous Section 1. (Page 27), was operated at volumetric efficiency 1.0 and 20°C. Wort of specific gravity 1.045 was employed throughout the experiment.

ii. The Wort

Unhopped wort of specific gravity 1.045 was prepared from a malt extract syrup, batch No. 1256, which was supplied by EDME Ltd., The analysis of the malt extract was forwarded to the author by EDME Ltd.

Analysis of Batch No. CME 1256.

Extract lbs per 224 lbs	70.4
Colour, 10% solution, 25 mm EBC	7.0
α D on sample	9.0
Diastatic Activity	4.0
Total sugars	73.7 per cent
Glucose and Fructose	8.0 per cent
Sucrose	4.8 per cent
Maltose	24.7 per cent
Maltotriose	7.7 per cent
Total fermentable sugars	45.2 per cent
Non-fermentable sugars	28.5 per cent
Protein	4.73 per cent
Total nitrogen	0.756 per cent
Permanently soluble nitrogen	0.714 per cent
α - amino nitrogen	0.152 per cent
Minerals	1.30 per cent
Water	20.3 per cent

iii. The yeast culture

The tower fermenter was inoculated with a culture of CFCC 1

(NCYC 1026), a brewing strain of Saccharomyces cerevisiae. Fifty colonies of the yeast were isolated at the commencement of operation and a similar number when the continuous operation was terminated.

The technique used to isolate the yeast colonies is described below.

iv. Preparation of isolates

Isolates of the yeast were prepared by diluting the culture yeast and preparing streak plates in the conventional manner. Fifty colonies isolated by this means were retained for investigation. The isolates were maintained on malt extract agar slopes held at $4^{o}C$.

v. Investigation of the yeast cultures

The following characteristics were investigated:

a. Giant Colony Morphology

The yeast cultures were inoculated onto the centre of malt extract agar plates prepared from dried malt extract (Oxoid Batch No. 6318). The plates were then incubated at $29^{o} \pm 1^{o}C$ for 14 days.

b. Cell shape

This was determined by microscopy.

c. Flocculence

Flocculence was determined by the method of Sharp (Greenshields et alia, 1972).

d. Spore formation

The yeast was cultivated on sodium acetate agar slopes (Fowell, 1952) for 7 days at $24^{o} \pm 1^{o}C$. The yeast was then suspended in distilled water and examined under the microscope.

e. Melibiose test

The medium for the test had the following composition:

Yeast extract powder (Oxoid)	1.5 g. litre
Peptone (bacteriological - Hopkins and Williams)	2.5 g. litre
Casein hydrolysate (Oxoid)	0.5 g. litre
Melibiose (B.D.H.)	40.0 g. litre
Bromocresol purple	0.005 g. litre

10 ml aliquots of this solution were pipetted into Universal bottles and Durham tubes inverted into the liquid. The bottles were then autoclaved at 0.7 kg/cm^2 for 10 minutes.

When cool the bottles were inoculated with the yeast isolates and incubated at $29^{\circ} \pm 1^{\circ}C$ for 3 to 6 days. A control fermentation with a strain of Saccharomyces uvarum was conducted with each batch of media. Fermentation was indicated by colour change from purple to yellow together with the presence of gas in the Durham tube. If no reaction was noted in the test bottle within 3 days of a positive reaction by the control, then the test was considered negative.

The following additional tests were conducted upon isolates of the yeast from the commercial tower fermenter.

f. Fermentation rate

The fermentations were carried out in round flat bottomed flasks maintained at $15^{\circ}C$ in a water bath, the necks of the flasks were plugged with tightly rolled cotton wool. The sterile malt wort (1.5 litres autoclaved at 1.05 kg/cm^2 for 15 mins) was inoculated

with 6 g. of freshly grown yeast. The flasks were not shaken whilst the fermentation was in progress and the present gravity was determined at intervals by removing samples with sterile pipettes.

g. Yeast yield

The yeast yield (as centrifuged wet weight) was measured at the termination of the fermentations described in f. above.

h. Attenuation gravity

The specific gravity of the beer from f. was measured 96 hours after pitching.

B. RESULTS

Results of investigations on CFCC 1

i. Giant Colony Morphology

All individual characteristics have been reported in the Appendix (Table A1).

The giant colony morphology of the parent yeast on malt extract agar is described below:

Diameter	-	16.8 (mean)
Surface	-	Smooth matt showing some concentric markings
Edge	-	Serrated
Shape	-	Circular
Elevation	-	a
Colour	-	cream

Isolates numbers 12 and 23 of the yeast from the tower fermenter showed some change in the form of the colony. Instead of the small serrations of the colony edge, number 12 showed large indentations of the margin so that the colony appeared to consist of a number of lobes. A similar morphology was noted with No. 23 though in this isolate the change was not as extreme. Isolate number 12 was mid brown and the surface appeared gritty with a matt finish. The colony was also the smallest recorded of any isolate. None of these latter changes were apparent in isolate number 23.

The individual values for the remaining parameters have been presented in the Appendix (Table A2), apart from those instances reported above all the characteristics of isolates were unchanged.

ii. Cell shape

There was no evidence of abnormality in the shape of the cells in any isolates, either from the inoculating yeast or the yeast removed from the tower fermenter after 4 months

continuous cultivation.

iii. Flocculence

The results indicated that no isolate has become completely non-flocculent but isolates numbers 35 and 37 did give flocculence values which were slightly low. The data were analysed by the 't' test.

	Parent Isolates	Test Isolates
Range of values	89 to 97	70 to 123
Mean	93.1	103.9
Standard Deviation	2.74	11.44
Variance	7.41	130.96
Degrees of Freedom	98	
t	6.49	
p	0.001	

The statistical analysis indicated that the difference between the means was very highly significant. However, the difference between the variances of the two samples was considerable and application of the Variance Ratio test (Bishop, 1969) indicated that the results of the 't' test were to be regarded with caution. Clearly the value of 't' = 6.49 obtained from the analysis was considerably greater than that ('t' = 3.46) presented in the Statistical Tables (Bishop, 1969) and so it was concluded that the results of the 't' test could still be regarded as valid. This was confirmed by application of the test devised by Cochran (1964).

It was concluded, therefore, that a significant increase in the flocculence of the yeast population had occurred during the period of continuous fermentation.

iv. Spore formation

All the isolates of the parent yeast were shown to produce spores quite readily. Sporulation was sparse in 17 isolates of

the yeast removed from the tower fermenter but none completely failed to produce spores.

v. Melibiose fermentation

All isolates of both the parent and the yeast from the tower fermenter were unable to ferment melibiose.

Results of investigation on CFCC 70 - S. cerevisiae (NCYC 1026)

i. Giant Colony Morphology

The information on individual colonies has been presented in the Appendix (Table A3).

The characteristics of the parent yeast colonies have been summarized below:

Diameter	-	17.0 mm (mean)
Surface	-	matt showing concentric and radial markings
Edge	-	serrated
Shape	-	circular
Elevation	-	a (conical projection present in the centre)
Colour	-	cream

None of the isolates of the yeast from the fermenter showed any changes in qualitative characteristics. The individual colony size of the parent and test isolates were plotted as a histogram (Fig. 5.). A near normal distribution was evident for both groups of isolates.

It was concluded that there had been no changes in the characteristics investigated.

The individual values for the remaining parameters have been presented in the Appendix (Table A4).

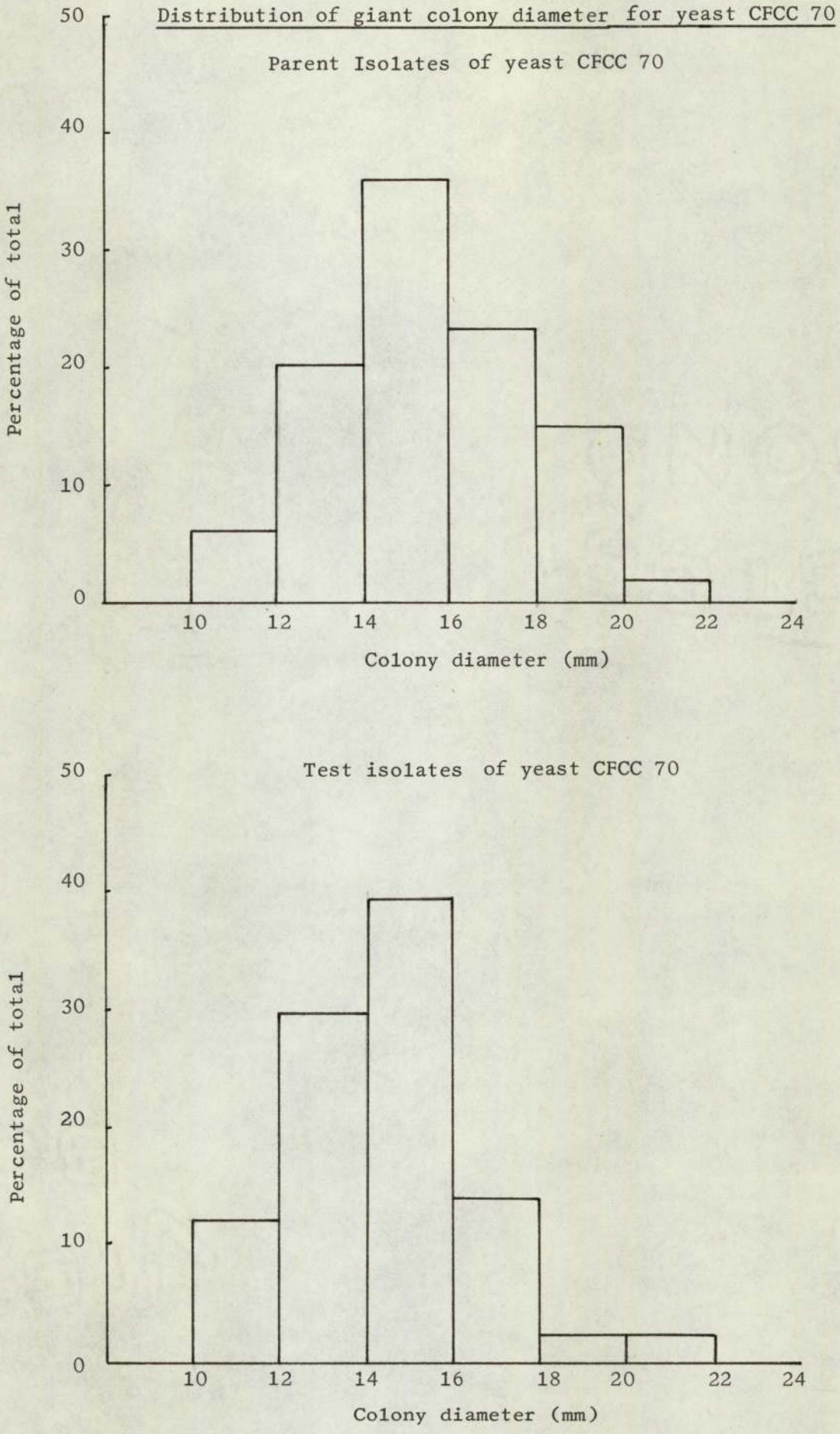
ii. Cell shape and size

This parameter has been reported as normal (n) or elongate (e).

All isolates consisted of cells with the normal round to ovoid shape.

The mean cell width of populations of each isolate was

Fig. 5.


Distribution of giant colony diameter for yeast CFCC 70
Parent Isolates of yeast CFCC 70
Percentage of total
50
40
30
20
10
0
10
12
14
16
18
20
22
24
Colony diameter (mm)
Test isolates of yeast CFCC 70
Percentage of total
50
40
30
20
10
0
10
12
14
16
18
20
22
24
Colony diameter (mm)

determined and the range of size reported below:

Parent Culture	3.8 to 6.2
Isolates from the fermenter	3.5 to 6.2

iii. Flocculence

Analysis of the values for flocculence of the parent culture and of the isolates of yeast from the tower fermenter are presented below:

	Parent Isolates	Test Isolates
Range of values	105 to 156	97 to 135
Mean	127.6	116.3
Variance	123.5	74
Standard Deviation	11.1	8.6
Degrees of Freedom	90	
Standard Error	1.718	1.217
t	5.52	
p	0.001	

Analysis of the data for the isolates as two populations show that there has been an overall decrease in flocculence as measured by this test. Application of statistical analysis indicated that the difference was significant at the 99.9% level of possibility.

The histogram (Fig. 6.) showed a normal distribution for isolates of the parent yeast and those of the yeast from the tower fermenter.

iv. Spore formation

The ability of isolates of the parent culture to form spores was demonstrated in all cases except two.

Eight of the isolates from the tower fermenter (Nos. 1, 2, 7, 8, 10, 16, 18, 19) failed to produce ascospores.

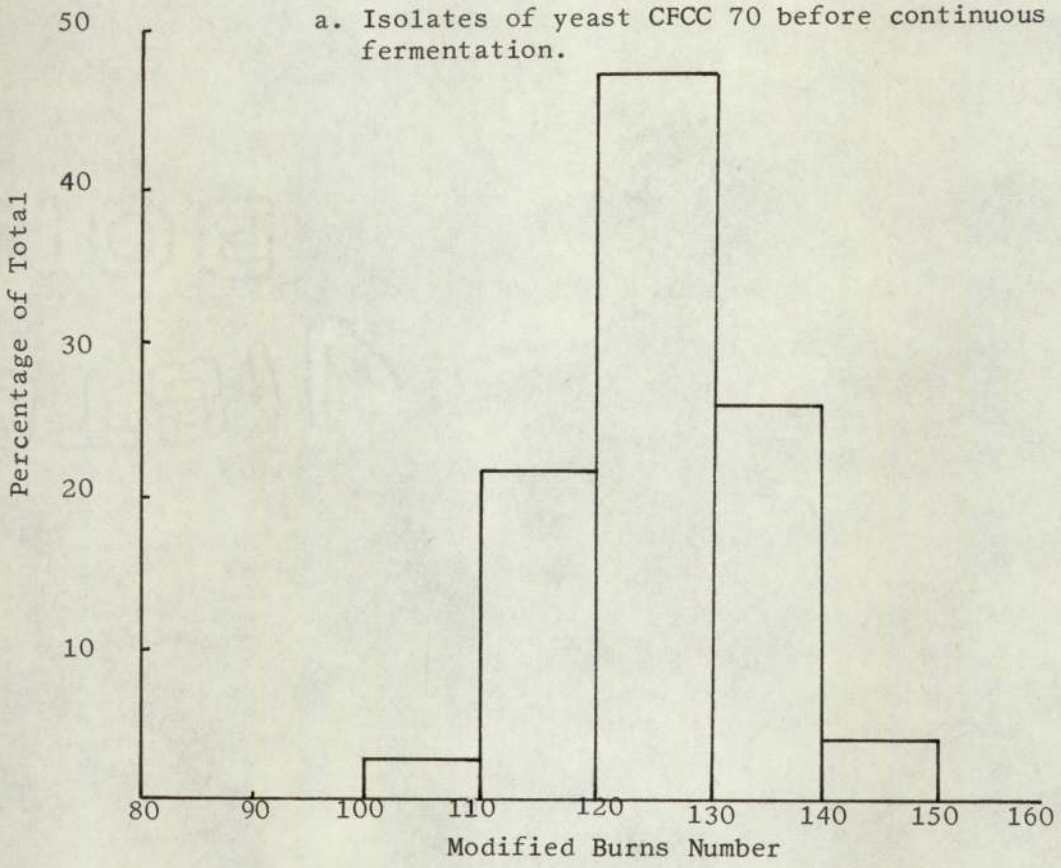
v. Melibiose fermentation

All isolates of both the parent culture and the yeast from the tower fermenter were unable to ferment melibiose.

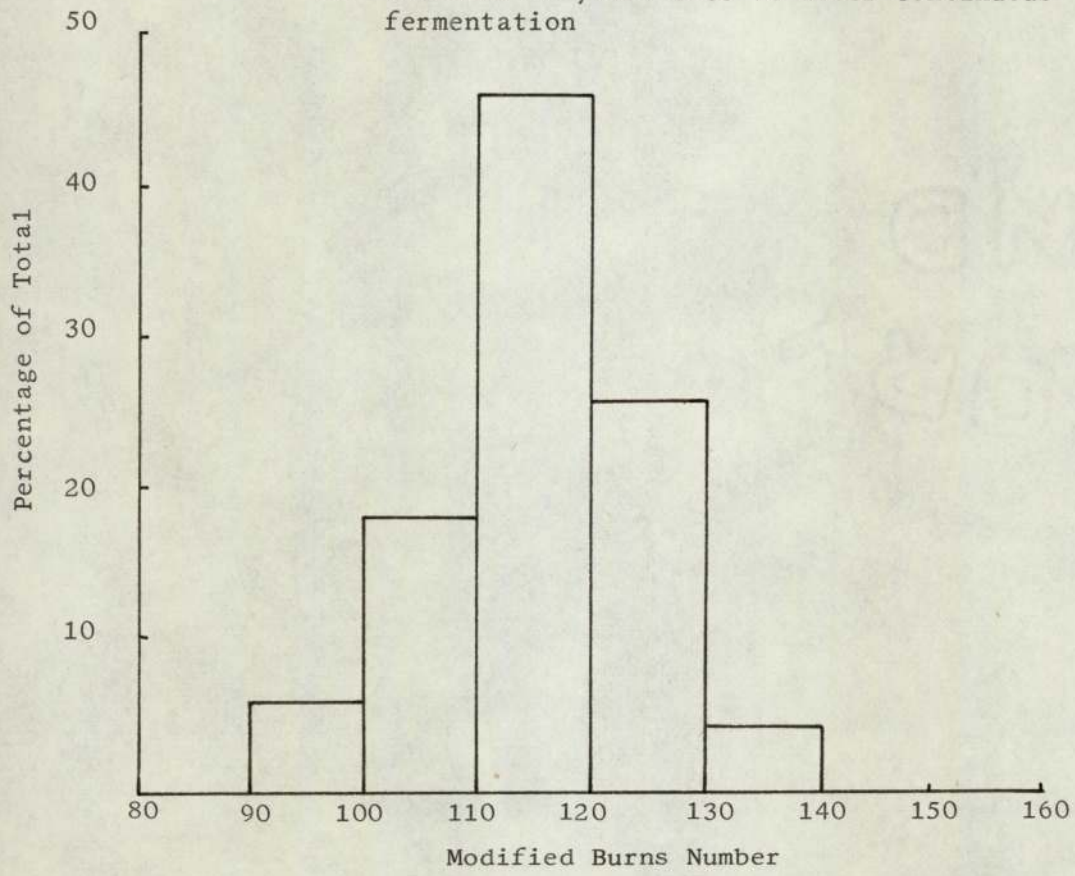
Figure 6

The distribution of values for flocculence of the yeast CFCC 70 before and after continuous fermentation

a. Isolates of yeast CFCC 70 before continuous fermentation.



b. Isolates of yeast CFCC 70 after continuous fermentation



vi. Fermentation rate

The measurement of this parameter revealed a wide range of values for fermentation rate by isolates of the parent culture. The range obtained with the isolates under test was narrower. Analysis of the results by the 't' test is presented below:

	Parent Isolates	Test Isolates
Range	0.0025 to 0.0050	0.0033 to 0.0044
Mean	0.00385	0.00378
Standard Deviation	0.00065	0.00029
Degrees of Freedom	98	
t	0.74	
p	0.1	

The individual values are presented in the Appendix (Table 4.).

The analysis indicated that the difference between the populations was not significant.

vii. Yeast yield

The results of measurement of yield of the parent culture were analysed by the 't' test.

	Parent Isolates	Test Isolates
Range	41 g to 57 g	40 g to 55 g
Mean	46.5 g	47.0 g
Standard Deviation	4.80	4.62
Degrees of Freedom	98	
t	0.51	
p	0.1	

There had been no significant change in this characteristic.

viii. Attenuation gravity

Analysis of the data upon this parameter is given below:

	Parent Isolates	Test Isolates
Range	1.007 to 1.010	1.0055 to 1.0115
Mean	1.0085	1.0075
Standard Deviation	0.0011	0.0016
Degrees of freedom	98	
t	1.01	
p	0.1	

The test isolates showed a considerable heterogeneity in this characteristic. The values obtained for the 3 test isolates (Nos. 6, 16, 17) fell outside the upper limit of the 95% confidence limits (Mean $\pm$ 2 S.Ds) and so might be considered to be mutants.

C. DISCUSSION AND CONCLUSIONS

The investigation of cell shape gave no indication of changes in this character. This is in good agreement with Thorne (1968, 1970) and so it would appear that this characteristic is stable. The production of elongated cell forms during continuous fermentation was reported by Brown and Hough (1965), but this change was found to be reversible, implying a physiological rather then genetic alteration.

Flocculence is a characteristic which is known to be under genetic control and mutation is generally in the direction of flocculence → non-flocculence. Thorne (1968) has reported that in a strain of Saccharomyces uvarum 7 of 48 isolates examined after continuous fermentation showed loss of flocculence and that a further 3 isolates were of indeterminate flocculence. Investigation of a second strain of Saccharomyces uvarum revealed that only one in fifty isolates showed this mutation but that a further three isolates were of indeterminate flocculence (Thorne, 1970).

The results presented on Page 52 indicated that a range of values for flocculence exists. The values obtained for the isolates of CFCC 1 after four months continuous fermentation indicate that no isolate showed a serious loss of flocculence and it seems reasonable to assume that none of these isolates had mutated. The values obtained after continuous fermentation showed a greater heterogeneity but the majority of values indicated an increase in flocculence which was confirmed by statistical analysis. These findings confirm those of Klopper et alia (1965) who reported an increase in flocculence during prolonged operation of a pilot scale tower fermenter.

Analysis of the results for yeast CFCC 70 revealed that a small

but statistically significant reduction in flocculence had occurred during continuous fermentation. However, this represented a quantitatively minor difference and a loss of flocculence to this extent should not seriously impair the performance of the fermenter under normal operational conditions.

The results obtained for yeast CFCC 1 and the findings for yeast CFCC 70 indicate that flocculence is a relatively stable characteristic in this strain of Saccharomyces cerevisiae.

The fear expressed by Thorne (1968) that loss of flocculence and subsequent depletion of the yeast mass within the fermenter has not been confirmed in practice. The experience of the author is that there is no problem in this respect, and to date there have been no reports to contradict these findings. Additionally modern development in the operation of tower fermenters has shown that it is advantageous to allow a level of yeast growth similar to that found in batch fermentation and when this procedure is followed it becomes necessary to allow yeast to escape with the beer, or even to deliberately remove the excess yeast. (Ault, Hampton, Newton and Roberts, 1969).

The investigation of the parent culture of CFCC 1 indicated that sporulation could be induced with reasonable ease. Some isolates of the yeast taken from the tower fermenter showed only sparse sporulation when tested, but it is significant that not one isolate had completely lost the ability to sporulate. In the case of the parent culture CFCC 70 it was found that some isolates showed only sparse sporulation on the media used. Eight isolates of the yeast from the tower fermenter failed to sporulate when tested. This in itself cannot be considered of great importance but Thorne (1970) has shown that loss of sporulation in

a strain of Saccharomyces uvarum was correlated with a reduction in the attenuation of the beer due to reduced ability of the yeast to ferment maltotriose. There was apparently no link of this nature in the case of CFCC 70 and so it seems reasonable to assume that the loss of sporulation ability by CFCC 70 cannot be regarded as an adverse change.

The ability to ferment melibiose is taken as the only reliable test to distinguish Saccharomyces uvarum from Saccharomyces cerevisiae (Lodder and Kreger van Rij, 1952). The yeasts used to inoculate the laboratory and commercial tower fermenters were both strains of Saccharomyces cerevisiae as confirmed by negative melibiose tests. It was demonstrated that no change in this characteristic occurred during the period of continuous fermentation. Thorne (1968) reported that one isolate of a strain of Saccharomyces uvarum apparently lost the ability to ferment melibiose after continuous cultivation and consequently could no longer be regarded as a member of the parent species. Work upon a different strain of Saccharomyces uvarum indicated no change in respect of melibiose fermentability (Thorne, 1970), and together with the findings presented in this report seem to indicate that interspecific changes from Saccharomyces cerevisiae to Saccharomyces uvarum or vice versa are in all probability infrequent occurrences and will not present a major hazard in the operation of continuous fermentation systems.

Analysis of the results for fermentation rate of the yeast CFCC 70 under conditions of batch fermentation have revealed that there was no adverse change when the isolates were considered as two populations. In fact the range of values obtained with the yeast isolated after continuous fermentation was narrower than the range for the isolates of the parent yeast.

Measurement of the yeast yield from batch fermentations by CFCC 70 isolates has shown that this characteristic was also stable and the results for the parent and tower yeast populations show very good agreement.

Attenuation of the beer produced in batch fermentation by the isolates of the parent and tower yeast strains of CFCC 70 showed a wide range of values. The isolates of yeast from the tower showed more heterogeneity than the isolates of the parent yeast. Three isolates of the tower yeast produced beers with a higher specific gravity than that found for any beer produced by the strains of the parent yeast, but several yeasts produced beers with a lower specific gravity. It is difficult to determine the effect of these changes upon the performance of the tower fermenter. However, if the mean value for the attenuation gravity of the beers produced by the tower yeast isolates is compared to the figure obtained for the parent yeast cultures, then it is apparent that the overall difference between the populations is only slight. Hence it seems reasonable to conclude that the extent of the changes discovered would not affect the fermentation rate within the tower fermenter to any great extent.

Assessment of the characteristics of giant colonies of the parent yeast and those isolates of the tower yeast indicate that only one culture (No. 13) isolated from the tower fermenter had altered. The colony produced by this yeast differed in all respects except the elevation of the colony, and so it seems reasonable to assume that this change represented a mutation. None of the isolates of CFCC 70 showed changes in colony morphology after continuous fermentation.

The results obtained with the yeasts from the tower fermenter

have shown that there have been no major changes in the yeast population after one year in continuous culture. A few changes in what may be regarded as characteristics of minor importance have been noted. Whether or not the observed changes may influence other more important properties of the yeast has not been established but certainly in CFCC 70 there was no impairment of brewing properties.

These findings are in contrast to the findings of Thorne (1968, 1970) who demonstrated a high degree of mutability in two brewery strains of Saccharomyces uvarum when these yeasts were cultivated continuously for long periods.

It may be expected that under conditions of low oxygen availability as in the laboratory tower fermenter, the rate of growth and hence the probability of mutations occurring will be lower. With respect to flocculence it may be anticipated that any mutant which showed loss of flocculence would tend to be washed out of the tower fermenter. The fact that only two isolates of CFCC 1 showed a notable reduction in flocculence seems to indicate that changes of this nature will not present a serious practical problem.

The lack of agreement between the findings of Thorne (1968, 1970) and those presented here raise the question of what is responsible for the difference. Some factors which undoubtedly exert an influence are the strains of yeast and the culture conditions employed.

It is known that brewing strains of Saccharomyces uvarum are genetically more labile than Saccharomyces cerevisiae (Hall, 1972). Under conditions of continuous culture a strain of lager yeast showed loss of flocculence after 6 weeks cultivation, but in a atrain of Saccharomyces cerevisiae this charcteristic was unchanged after

six months continuous cultivation (Hall, 1970).

The spontaneous mutation of lager yeast in use in a brewery was reported by Gyllang and Martinson (1972). A number of respiratory deficient mutants with increased sedimentation rates were isolated and it was found that many of these mutants had lost the ability to ferment maltotriose.

The normal batch practice ensures that gross changes in the yeast mass are avoided by employing fresh pure culture yeast every few (normally 5 - 7) fermentation cycles. However, it is widely accepted that in a continuously stirred fermentation system such as employed by Thorne (1968b, 1970) the proliferation of mutants is encouraged. The proportion of mutants in the culture will depend upon the relative growth rates of the mutant and culture yeasts and the frequency with which fresh mutations appear.

A culture of Saccharomyces cerevisiae in the tower fermenter provides a complete contrast. The inherent genetic stability is complemented by the slow growth rate of the yeast under the conditions within the tower fermenter.

Finally in Thorne's analysis of the parent culture a standard error was quoted for the estimation of the continuously variable characteristics but no mention was made of the number of estimations performed upon the parent yeast culture. In the foregoing work the continuously variable characteristics were determined for fifty isolates of the parent culture and the results indicated that even with a pure culture of yeast a wide range of characteristic values may be given. It seems therefore that an analysis of a continuously varying characteristic should include an extensive investigation of the parent culture in order to establish the range of 'normal' values.

Ault et alia (1969) suggested that there was good circumstantial evidence for the stability of the yeast NCYC 1026 in the tower fermenter and the findings of the author confirm that observation.

SECTION 3

THE SELECTION OF YEASTS FOR THE TOWER FERMENTATION OF LAGER BEER

INTRODUCTION

During the fermentation of brewers wort by yeast, various by-products are formed in addition to ethanol and carbon dioxide. Many of these are present only in very small quantities, but they have a pronounced effect upon the flavour of the beer. In general, the same aroma components are present in beer, wine and distilled beverages, but within the same types of beverage the amounts of certain substances can vary considerably (Suomalainen, 1971). To date over 100 aroma compounds, including alcohols, fatty acids, aldehydes, sulphur compounds, esters and vicinal diketones have been identified.

Beer normally contains up to 200 ppm of higher alcohols of which isoamyl alcohol (20 - 95 ppm), phenylethyl (6 - 50 ppm), 'active' amyl (8 - 30 ppm) and iso-butyl alcohols (4 - 30ppm) are the major components (Thorne, 1966).

Acetic and lactic acids are the major fatty acids in beer and may cause sourness if present in excessive amounts. The taste threshold for lactic acid is quoted as 200 ppm, double that of acetic acid (Harrison, 1963). Butyric and valeric acids are also considered to be major flavour components (Thorne, 1966).

An unpleasant sweetness in beer may be due to an excess of ethyl, iso-butyl and iso-amyl acetates. However, small quantities may not necessarily be recognized as 'estery' but may merely modify other flavours.

Hydrogen sulphide can be sensed in beer at concentrations of 0.005 ppm though the typical H_2S smell is not detected below 0.05 ppm. The other major sulphur compounds, mercaptans, are evil smelling substances but if present in minute amounts may be beneficial to aroma.

The principal aldehyde found in beer is acetaldehyde. At the end of primary fermentation, its concentration may be from 10 to 35 ppm

but the level declines to between 2 and 9 ppm during storage. The presence of acetaldehyde is characteristic of 'green' or 'young' beer and its disappearance is one of the most important changes which occur during the maturation process.

Rosculet (1970) has presented an extensive review of publications relating to the aroma and flavour of beer.

The contribution of vicinal diketones to beer flavour and its origin in fermentation will now be discussed more fully.

The vicinal diketones

The vicinal diketones normally found in beer are diacetyl and 2, 3-pentanedione. Acetoin is linked with these compounds although it is not a diketone. The chemical formulae are indicated below.

Diacetyl	$CH_3CO.CO.CH_3$
2.3-pentanedione	$CH_3CO.CO.C_2H_5$
Acetoin	$CH_3CO.\underset{OH}{\overset{H}{C}}.CH_3$

The flavours and odours of diacetyl and 2.3-pentanedione have been described as 'butter', 'honey' or 'toffee', but the flavour perceived depends upon the concentration of the compounds in the beer. Excess acetoin is said to contribute a 'musty' flavour to beer.

Attempts have been made to determine the taste thresholds of these compounds in beer but the picture is complicated by varying ability of individuals to detect the substances. However, it is generally accepted that the threshold for lager beers is between 0.1 - 0.2 ppm (Drews et alia, 1962; Harrison, 1970).

Wainwright (1973) has presented an extensive review of diacetyl and its importance in brewing, and so anything further than a statement of the main points would be superfluous.

The amount of diacetyl found in beer is the result of the interaction of three main processes:-

i. The formation of acetohydroxy acids.

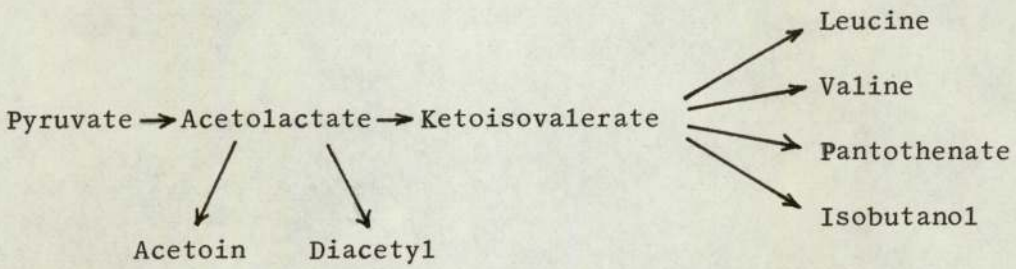
ii. The conversion of acetohydroxy acids to diketones.

iii. Removal of diketones.

The formation of acetohydroxy acids

Strassman et alia (1958) demonstrated that in Saccharomyces cerevisiae acetolactate is formed as an intermediate in valine biosynthesis.

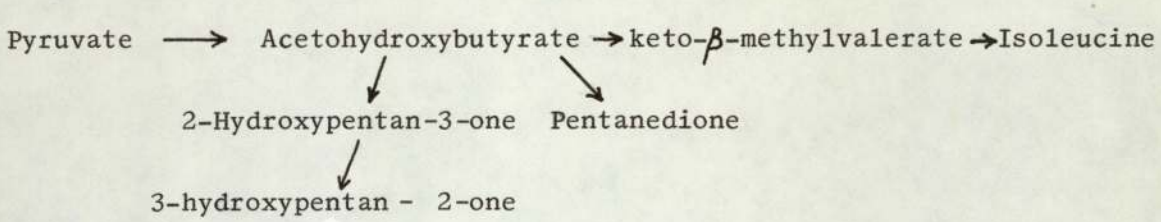
Fig. 7. Biosynthesis of diacetyl (Wainwright, 1973)



Yeast appears to possess only one acetohydroxy acid synthetase which is therefore involved in the synthesis of both acetolactic and acetohydroxybutyric acids (see Fig. 8.). The amino acid valine is known to specifically suppress the formation of acetolactic acid by yeast (Chuang & Collins, 1968; Portno, 1966b), but when valine is added to a fermentation the formation of acetohydroxybutyric acid is enhanced (Scherrer, 1972). Thus it appears that valine does not exert a direct influence upon acetohydroxy acid synthetase. At present the mechanism by which valine controls acetolactic acid formation is obscure but it is certainly complicated (Wainwright, 1973).

The pathway to the formation of pentanedione has been summarised in Fig. 8. below. Isoleucine inhibits the formation of acetohydroxybutyric acid by a similar mechanism to that which controls acetolactic acid.

Fig. 8. Biosynthetic origin of pentanedione (Wainwright, 1973)



Brenner (1970) has suggested that acetohydroxy acid formation is correlated with the vigour of fermentation. Thus the use of high temperatures and pitching rates increased the formation of acetohydroxy acids (Portno, 1966a).

Chuang and Collins (1968,1972) believe that there is an alternative pathway (Fig. 9.) for the production of diacetyl by Saccharomyces cerevisiae. The indications are, however, that if the pathway does exist only small quantities of diacetyl are synthesised by this route.

The conversion of acetohydroxy acids to diketones

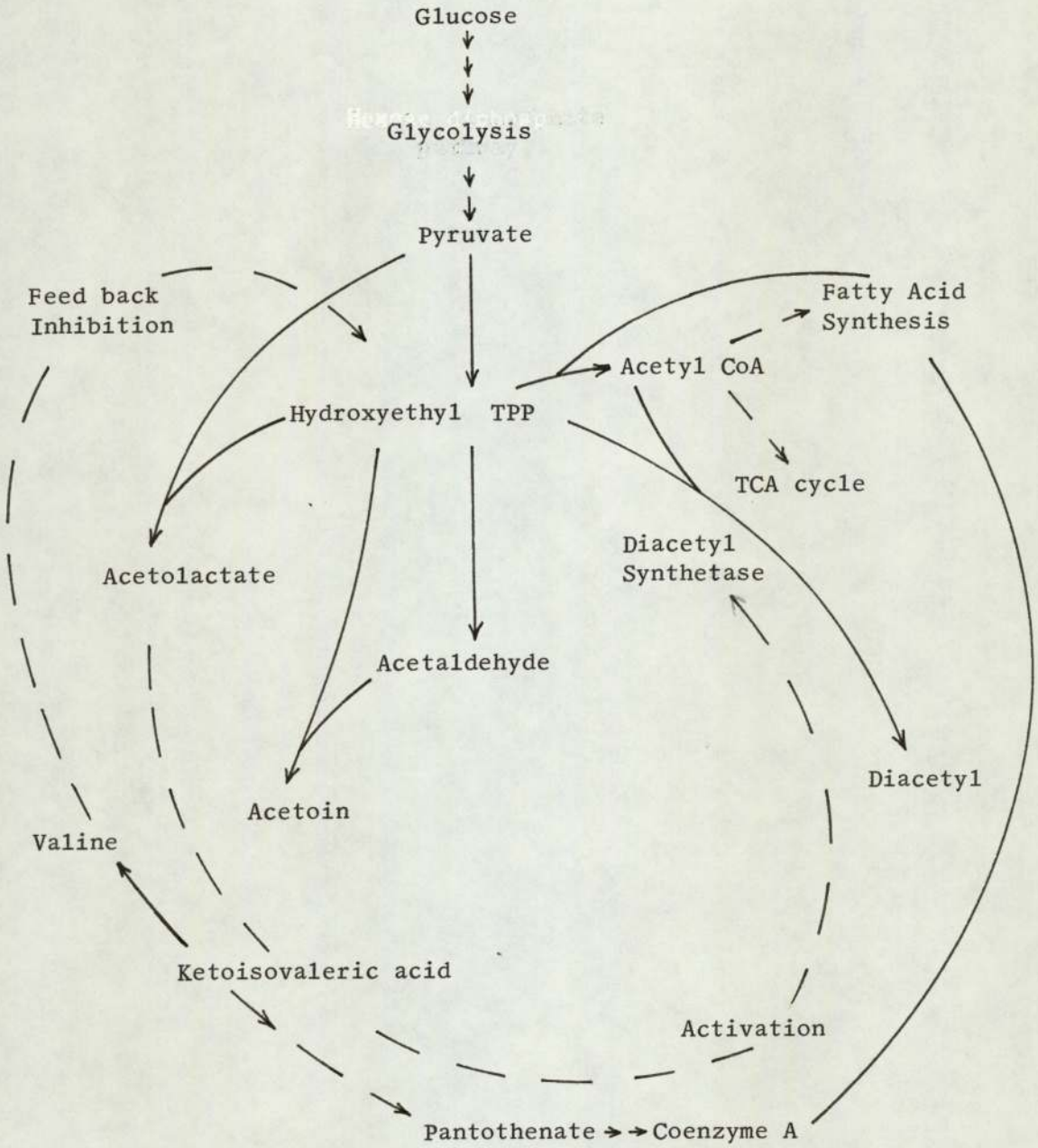
It is believed that the majority of diacetyl formed during fermentation arises as a result of the spontaneous oxidative decarboxylation of extra-cellular acetolactate (Inoue et alia, 1968c). This mechanism only operates when the oxidation reduction balance of the medium is above rH 10 (Inoue et alia, 1968b).

The decomposition mechanism can alter at very low pH values (below pH 2) to give acetoin (Inoue et alia, 1968a), but at pH 4 both acetoin and diacetyl are formed (Haukeli and Lie, 1971). Ronkainen et alia (1970) have reported that between pH5 - 6 acetolactic acid may decompose to yield diacetyl and formic acid though the mechanism of the formation of the latter is not understood.

The removal of diketones

The level of diacetyl in beer depends upon the rate and extent to which it is removed by the yeast. Liebs et alia (1970) demonstrated

Fig. 9. Diacetyl Synthesis in Saccharomyces cerevisae (Chuang & Collins, 1972).



by radioactive studies that Saccharomyces uvarum metabolised diacetyl via acetoin to butanediol.

In healthy yeast the rate of metabolism is rapid but temperature has a major influence. At 0°C the reduction of diacetyl takes several weeks but at 20°C the removal may be complete in 1 - 2 hours. (Ishibashi et alia, 1969). Early separation of the yeast from the beer may prevent reduction of diacetyl.

Thompson et alia (1970) studied the removal of diacetyl from beer by a bacterial diacetyl reductase. The enzyme requires NADH and is rapidly inactivated unless protected with gelatin. Ishibashi et alia (1969) found that in vitro at pH 4.2 the reduction of diacetyl was limited by the availability of NADH which is unstable under acidic conditions. However, there is no evidence to indicate that yeast possesses this enzyme, and it is believed that alcohol dehydrogenase is responsible for the reduction of diacetyl by yeast (Chuang & Collins, 1968).

Diacetyl in brewing

The amount of diacetyl in beer is the net result of the three processes which have been outlined previously.

The acetohydroxy acids are formed early in fermentation during the phase of growth and rapid metabolism. Brenner (1970) has suggested that if a beer contains a high level of diacetyl it is usually because the rate of diacetyl removal has slowed or stopped. This may occur as the result of early separation of the yeast by flocculation or because of a slowing of metabolic activity.

The use of low temperatures as in traditional lagering practice slows the spontaneous conversion of acetohydroxy acids and the subsequent metabolism of the diketones. The normal method of rectifying lager beers with high diketone contents is to add a portion

of fermenting wort - 'krausening' or some fresh yeast.

Other factors known to influence the levels of diketones in beer include yeast strain (Kringstadt & Rasch, 1966; Portno, 1966a; Wellhoener, 1967) and pitching rate (Portno, 1966a).

The effect of aeration depends upon the stage in the fermentation at which the oxygen is introduced. Aeration of the wort prior to pitching does not normally increase the level of diketones in the beer, but continuous aeration of the fermenting wort can lead to elevated levels of diketones in the beer (Portno, 1966b). Exposure of the beer to air at the end of primary fermentation can cause the yeast to grow again and in consequence produce acetohydroxy acids (Brenner, 1970).

Insufficient aeration can be just as hazardous if, as a result, the fermentation is sluggish (Brenner, 1970).

The influence of wort composition upon the levels of vicinal diketones has been investigated by many workers. The use of low nitrogen worts containing a large percentage of adjunct may result in a beer containing increased levels of diacetyl (Spaeth & Trabattoni, 1967). The removal of diacetyl at the end of fermentation may be ensured by the use of a highly fermentable wort containing sufficient assimilable nitrogen (Wainwright, 1973).

Diacetyl and tower fermentation

The published literature relating to tower fermentation indicates that relatively high levels of diketones are present in the young beer (Klopper at alia, 1965; Ault et alia, 1969). At low wort flow rates the production of diketones and their precursors is complete by about the mid point of the fermentation, and then a progressive reduction in the levels of these components is seen. At high wort flow rates there is a gradual increase in the amount of these compounds from the base to the head of the fermenter (Ault et alia, 1969).

If the levels of diacetyl and related substances are excessive, then it may be necessary to store the beer for lengthy periods to ensure adequate reduction of these compounds. Consequently many of the advantages of continuous fermentation would be neutralised. Ideally the storage period should be dispensed with completely, but it is essential that any period required for flavour development be kept to a minimum. Thus if beer could be produced with the necessary low levels of vicinal diketones, the period of lagering during which these compounds are removed from the beer by the yeast could be drastically reduced or ultimately dispensed with completely, provided the correct balance of flavour compounds is maintained.

Although at least one major brewing company in the United Kingdom has successfully employed the tower fermenter for the production of ale, attempts to produce lager beer by this method of continuous fermentation have shown that the control of the levels of vicinal diketones in the beer is a major problem (Starkie, 1971). Therefore an investigation was initiated in an attempt to establish the relative influence of yeast strain, wort flow rate, temperature and aeration upon the levels of these compounds.

Lager beer is mainly produced in batch fermentations employing strains of Saccharomyces uvarum, with characteristics suited to the particular conditions of that type of fermentation. Batch fermentation practice has favoured the use of yeasts which remain dispersed in the beer and only flocculate in the late stages of the fermentation. For this reason many of the yeasts which have been used with success in batch fermentations may not be suitable for use in a tower fermentation system, because the conditions within a tower fermenter are such that only yeasts which aggregate to form relatively large and dense flocs remain in the fermenter.

This investigation was undertaken with the object of selecting

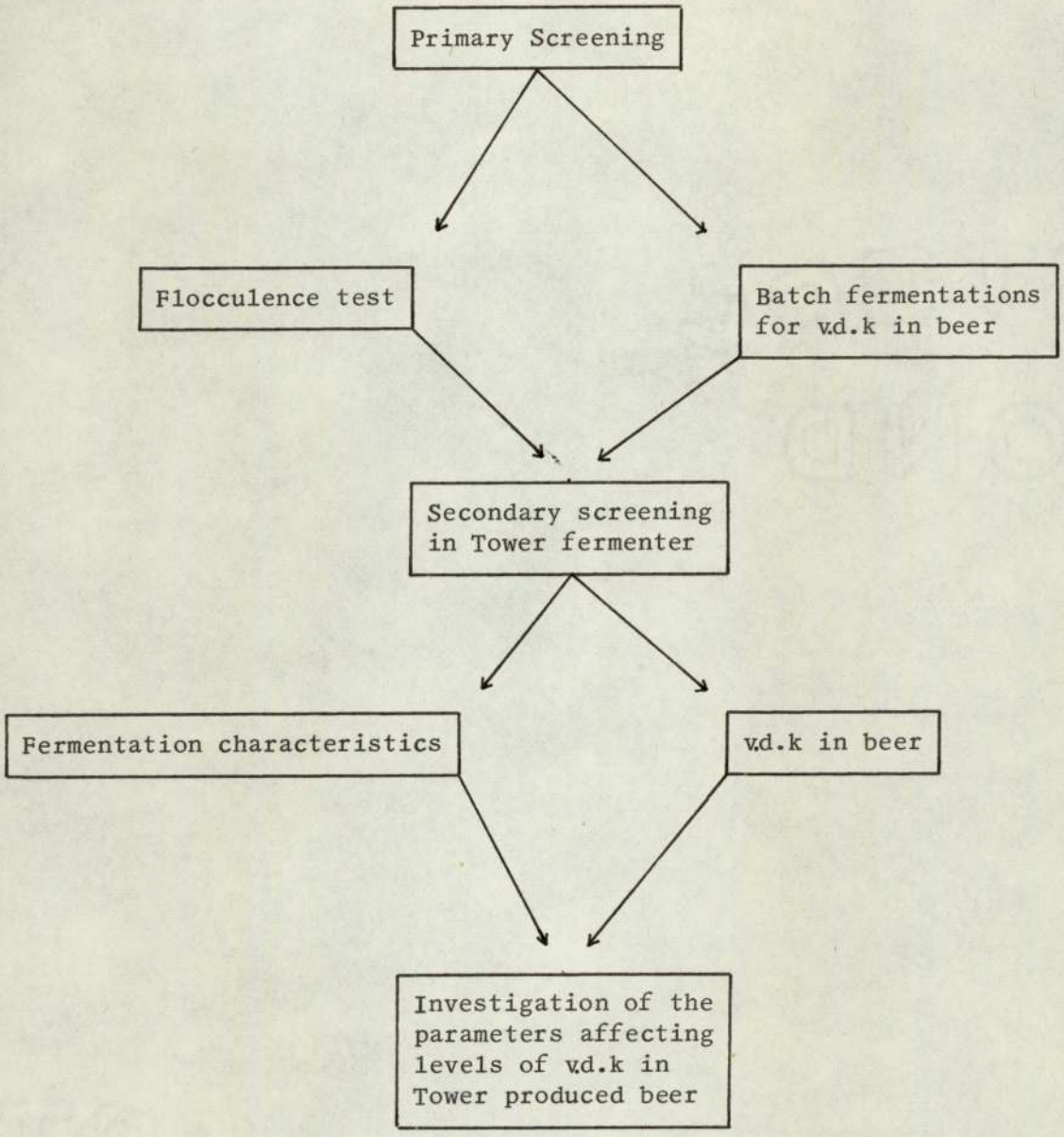
yeasts which could be used for production of lager beer by continuous tower fermentation.

A technique to select ale yeasts for the tower fermenter was developed in the fermentation technology laboratory at the University of Aston (Greenshields and Smith, 1971). The technique which involved a two stage screening procedure was adapted for this investigation. Initially the flocculation characteristics of the yeasts were assessed by means of a modified Burns sedimentation test (Greenshields et alia, 1972). An additional test to determine the levels of vicinal diketones in batch produced beer was incorporated into the first stage testing.

The yeasts selected for further investigation were then cultured in the laboratory tower fermenter and the beers analysed for vicinal diketone content. Further tests were conducted with two yeasts in an attempt to elicidate the operational parameters which influence the levels of vicinal diketone in tower fermented beer.

The scheme of this investigation has been presented overleaf.

Scheme for the selection of lager yeasts for continuous tower fermentation



PRIMARY SCREENING

The strains of yeasts used for the production of lager beers by batch fermentation belong to the species Saccharomyces uvarum. Accordingly a number of commercial brewing strains of the species were obtained by the A.P.V. Company Limited, and forwarded to the author. Details of these yeasts have been tabulated below.

A. MATERIALS AND METHODS

i. The yeast cultures

The yeasts, all strains of Saccharomyces uvarum were obtained from the sources listed below, by the A.P.V. Company Limited, Manor Royal, Crawley, Sussex and forwarded to the author. These yeasts were held in the continuous fermentation culture collection (CFCC).

CFCC 39 Alfred Jørgensen Gaeringsfysiologisk Laboratorium A/S Copenhagen.

CFCC 49 Bottom type 8, ex. A.P.V. Company Limited.

CFCC 50 Bottom type 41, ex. A.P.V. Company Limited.

CFCC 51 Bottom type 11, ex. A.P.V. Company Limited.

CFCC 52 Alfred Jørgensen Gaeringsfysiologisk Laboratorium A/S Copenhagen.

CFCC 65 Y. 11, ex. Department of Microbiology, University of Strathclyde.

CFCC 73 0616 ex. Versuchs und Lehranstalt fur Brauerei in Berlin.

CFCC 75 0613 ex. Versuchs und Lehranstalt fur Brauerei in Berlin.

CFCC 76 0611 ex. Versuchs und Lehranstalt fur Brauerei in Berlin.

CFCC 77 0614 ex. Versuchs und Lehranstalt fur Brauerei in Berlin.

CFCC 79 0623 ex. Versuchs und Lehranstalt fur Brauerei in Berlin.

CFCC 80 0620 ex. Versuchs und Lehranstalt fur Brauerei in Berlin.

CFCC 82 No. 233 Dansk Gaerings Industri.

CFCC 83 No. 234 Dansk Gaerings Industri.

ii. Storage of the yeast cultures

Upon receipt all cultures were streaked out on malt, yeast extract, glucose, peptone (MYGP) (Wickerham 1951) agar plates prepared as below:

Malt Extract (Oxoid)	3 g. litre
Yeast Extract (Oxoid)	3.g. litre
Glucose	10 g. litre
Mycological Peptone (Oxoid)	5 g. litre
Agar	2 g. litre

The whole was warmed over a bunsen burner to dissolve the agar and then 250 ml. aliquots placed in conical flasks and autoclaved at 1.05 kg/cm^2 for 15 minutes. The sterile agar was poured into 90 mm. petri plates.

When inoculated the plates were incubated at 29 - 30°C until single colonies of approximately 1 mm. diameter had developed. Colonies isolated in this way were used to inoculate M.Y.G.P. agar slopes which were incubated 3 - 4 days and then stored in a refrigerator at 4°C. Subcultures were made routinely every 6 months, or after the master culture had been opened three times.

iii. Malt Extract

The malt extract, batch number LME 849, was supplied by Edme Limited, Mistley, Essex. The analysis presented below was carried out by Edme Limited and the results forwarded to the author.

Extract brewers lb. per 224 lb.	70.7
Colour, 25 mm E.B.C. 10% soln.	4.5
% Fermentability (apparent)	64.2

Total sugars	73.0 %
Glucose and fructose	13.3 %
Sucrose	1.0 %
Maltose	31.7 %
Maltotriose	8.8 %
Non Fermentable Sugars	18.2 %
Protein	5.75 %
Mineral Matter	1.28 %
Water	20.0 %
Total Nitrogen	0.921%
Permanently Soluble Nitrogen	0.795%
Lundin A fraction	0.319%
Lundin B fraction	0.105%
Lundin C fraction	0.497%
α - amino nitrogen	0.200%

iv. Preparation of Inoculum

The inoculum was prepared from the master culture by growing up in 10 ml. and then 100 ml. quantities of sterile malt wort at 30°C. The fermentation of the 100 ml. quantity of wort was allowed to run to completion when the yeast settled out. All but 20 ml. of the supernatant beer was decanted off, and the yeast suspended in the remaining beer. This suspension was used as the inoculum for the test fermentation.

v. Preparation of Wort

The malt wort of specific gravity 1.045 was prepared from malt extract syrup supplied by E.D.M.E. Limited. 1000 ml. aliquots were placed in 2 litre flasks and autoclaved at 1.05 kg/cm^2 for 15 minutes.

vi. Attenuation Limit

The wort of original gravity 1.045 was inoculated with 1.5 g. yeast (centrifuged wet weight) per 100 ml of wort. The yeast, CFCC 83,

had been freshly grown from the master culture by successive transfers in wort of original gravity 1.045.

The temperature was controlled at 20°C and the fermentation allowed to continue for 7 days.

Analysis:

	Wort	Beer
Specific gravity	1.045	1.007

vii. Batch Fermentations

Wort of original gravity 1.045 was fermented at 15°C for 168 hours. The specific gravity of the beer and the total vicinal diketones (v.d.k.) were measured at the end of this time.

viii. Storage of Beer

The beer produced in the test fermentations was stored at 4°C in screw cap glass bottles for a period up to 28 days. The level of total v.d.k. in each beer was determined once every 7 days over this storage period.

ix. Flocculence Test

The flocculence of the various yeast strains was determined by Sharp's modification of the Burn's method (Greenshields, Yates, Sharp and Davies, 1972).

x. Specific Gravity

This was determined at 15.5°C by the use of hydrometers (W. Reeves and Company Limited), or specific gravity balance (Westphal type, Stanton Instruments Limited).

xi. pH

A Pye Model 79 pH meter was used.

xii. Total Acidity

0.1N. NaOH was titrated against a 10 ml. sample of degassed beer, using phenolphthalein as an indicator.

xiii. Yeast Count

The yeast content of the beer was determined by microscopy using a haemocytometer (Thoma pattern).

xiv. Vicinal Diketones

Vicinal diketones were determined by the recommended method of the Institute of Brewing (Ault, 1968).

B. RESULTS

i. Batch fermentations

Wort of original gravity 1.045 was fermented at 15°C for 7 days. The analyses of beers are presented below in Table 1.

Table 1.

Analysis of the beers produced by various yeasts after 168 hr. fermentation

Beer No. *	Present Gravity	Total v.d.k. (ppm)
39	1.0103	1.320
49	1.0085	0.700
50	1.0091	0.720
51	1.0092	0.280
52	1.0080	0.220
65	1.0080	0.105
73	1.0070	0.270
75 a	1.0075	0.209
b	1.0070	0.208
76 a	1.0075	0.405
b	1.0070	0.200
77 a	1.0067	0.109
b	1.0090	0.355
79 a	1.0075	0.295
b	1.0070	0.293
80 a	1.0075	0.120
b	1.0075	0.100
82 a	1.0070	0.075
b	1.0073	0.100
83 a	1.0070	0.040
b	1.0070	0.100

Note for Table 1.

* The identification number of the beer was derived from the CFCC number of the yeast e.g. Beer No. 39 was produced with yeast CFCC 39.

ii. Flocculence test

The flocculence of each yeast was assessed by Sharp's modification of the Burn's test. The results are presented below:

Table 2.

Flocculence of the yeast cultures

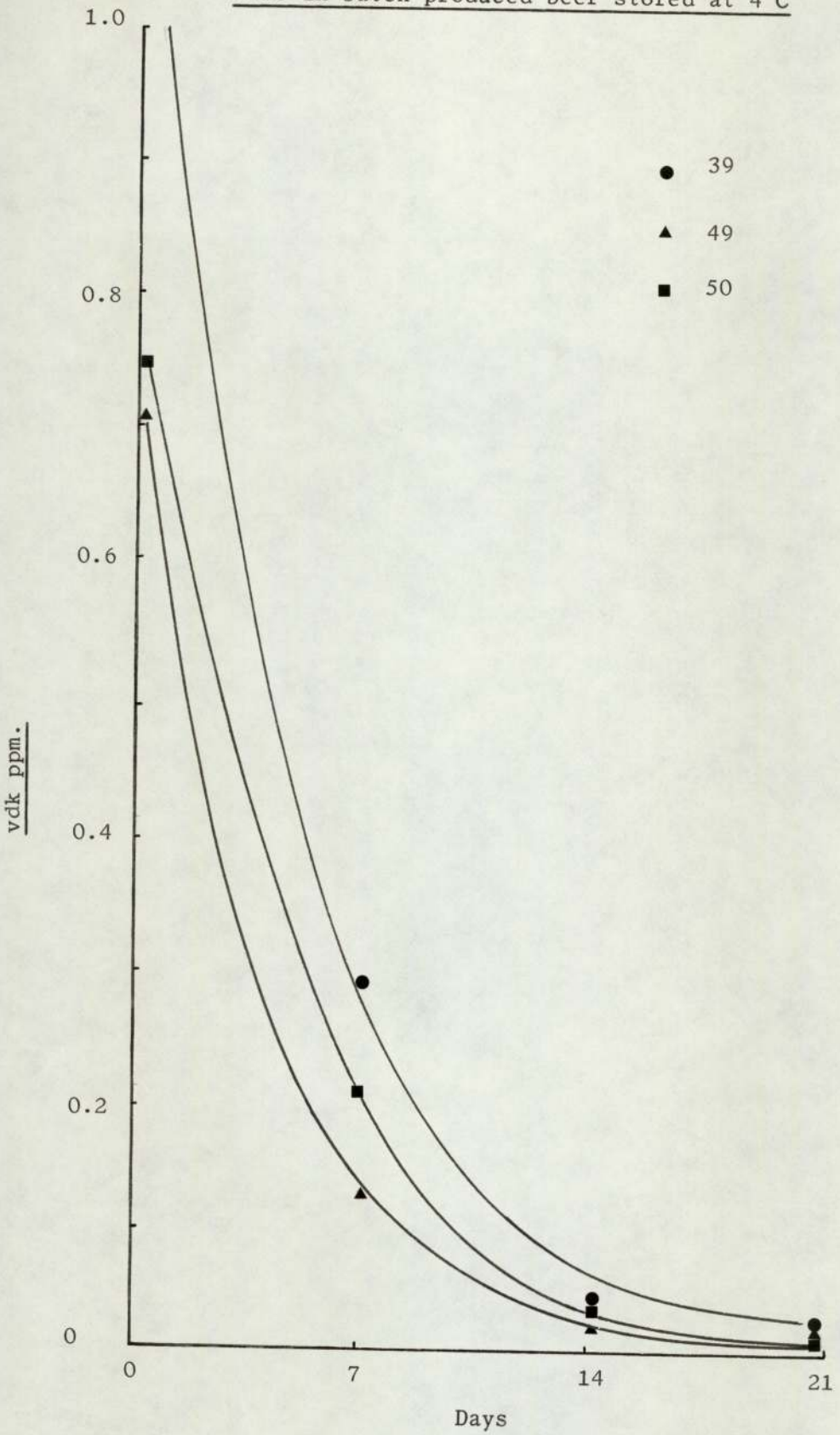
Yeast culture	Modified Burn's number
39	78
49	3
50	9
51	10
52	60
65	0
73	94
75	46
76	0
77	4
79	4.5
80	35.3
82	0
83	39.8

iii. Storage of beer

The effect of storage upon the levels of vicinal diketones in the beer is illustrated in Graphs 5 - 9.

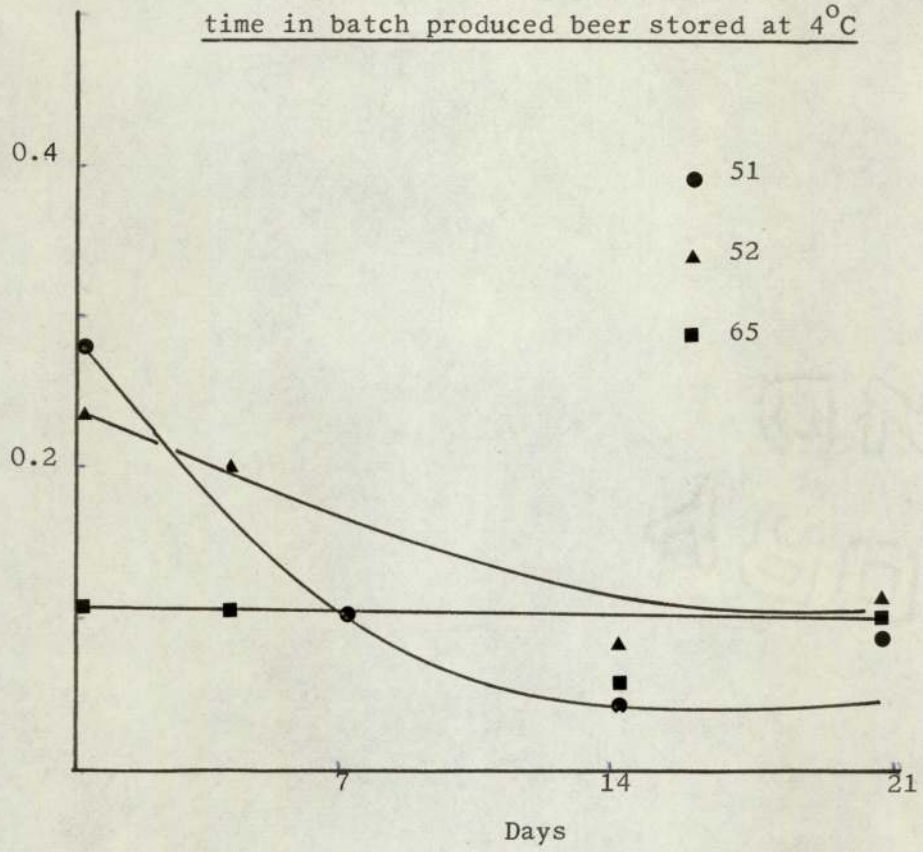
Graph 5

The change in levels of vicinal diketones with time in batch produced beer stored at $4^{o}C$

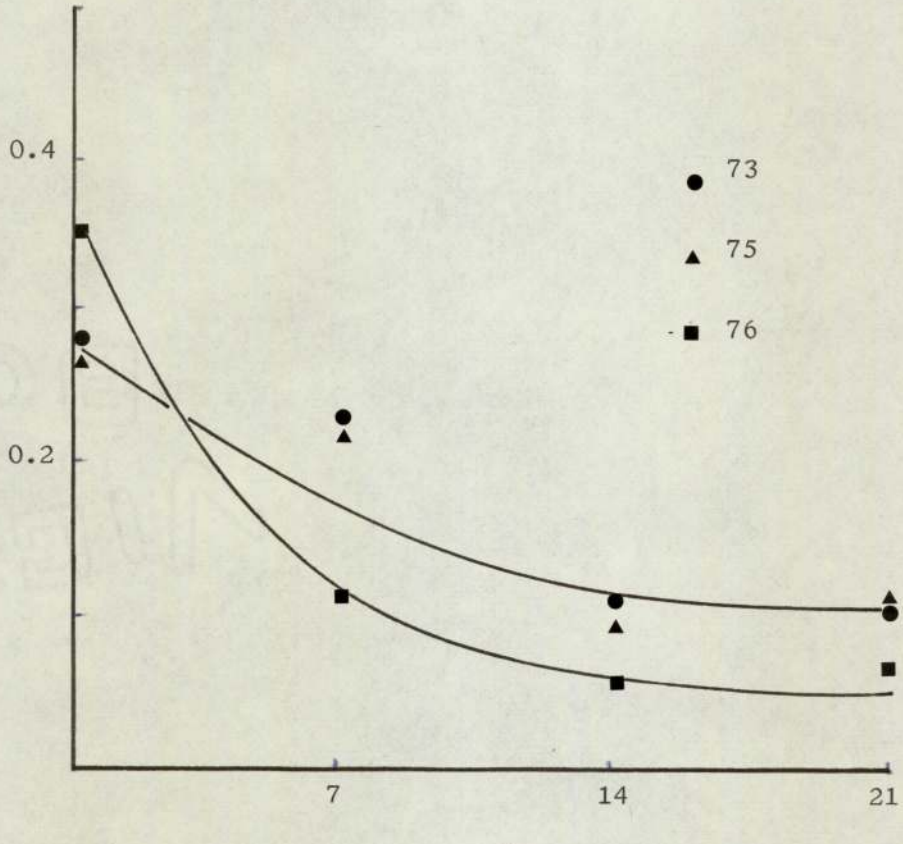


Graph 6

The change in levels of vicinal diketones with time in batch produced beer stored at 4°C

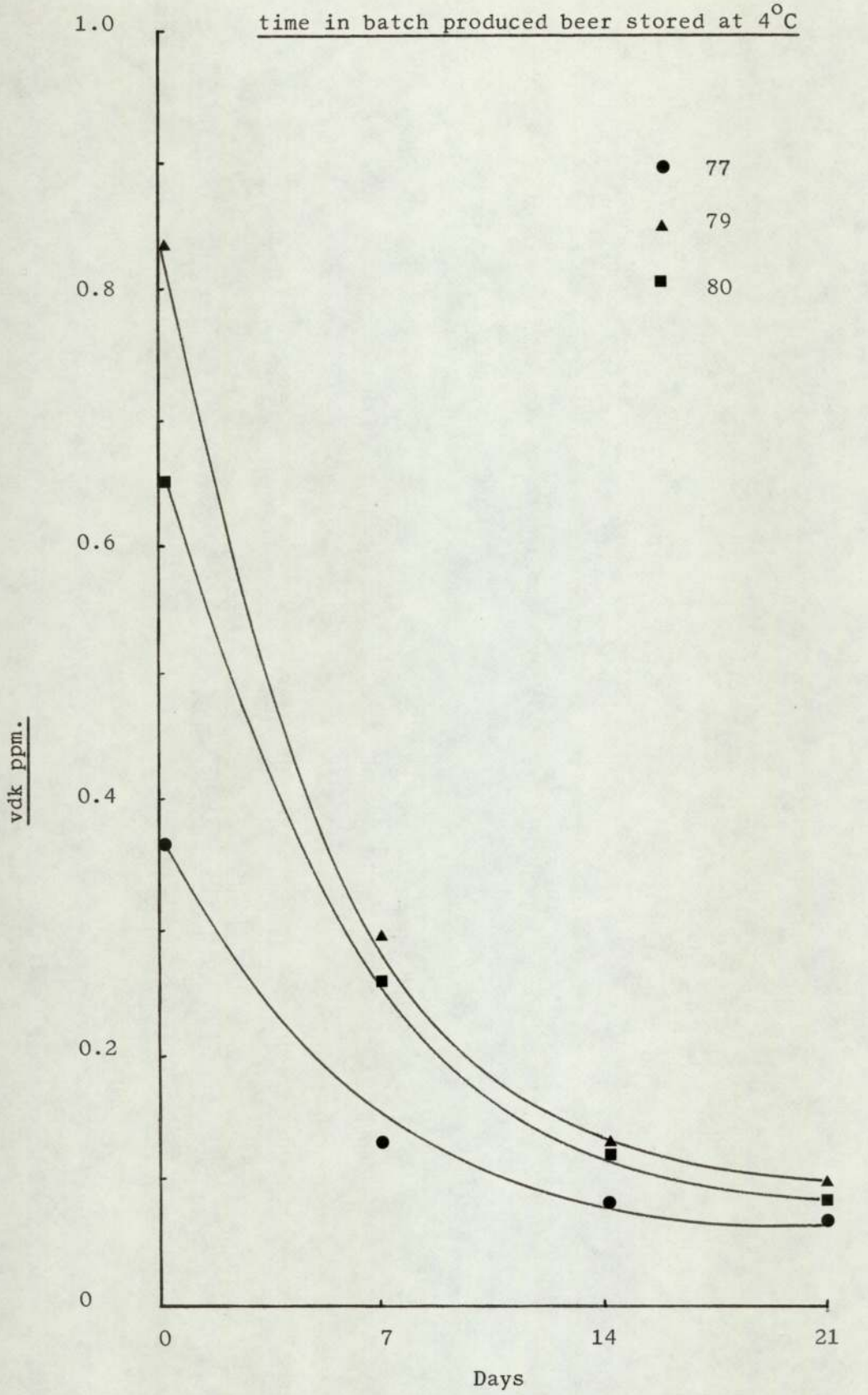


Graph 7



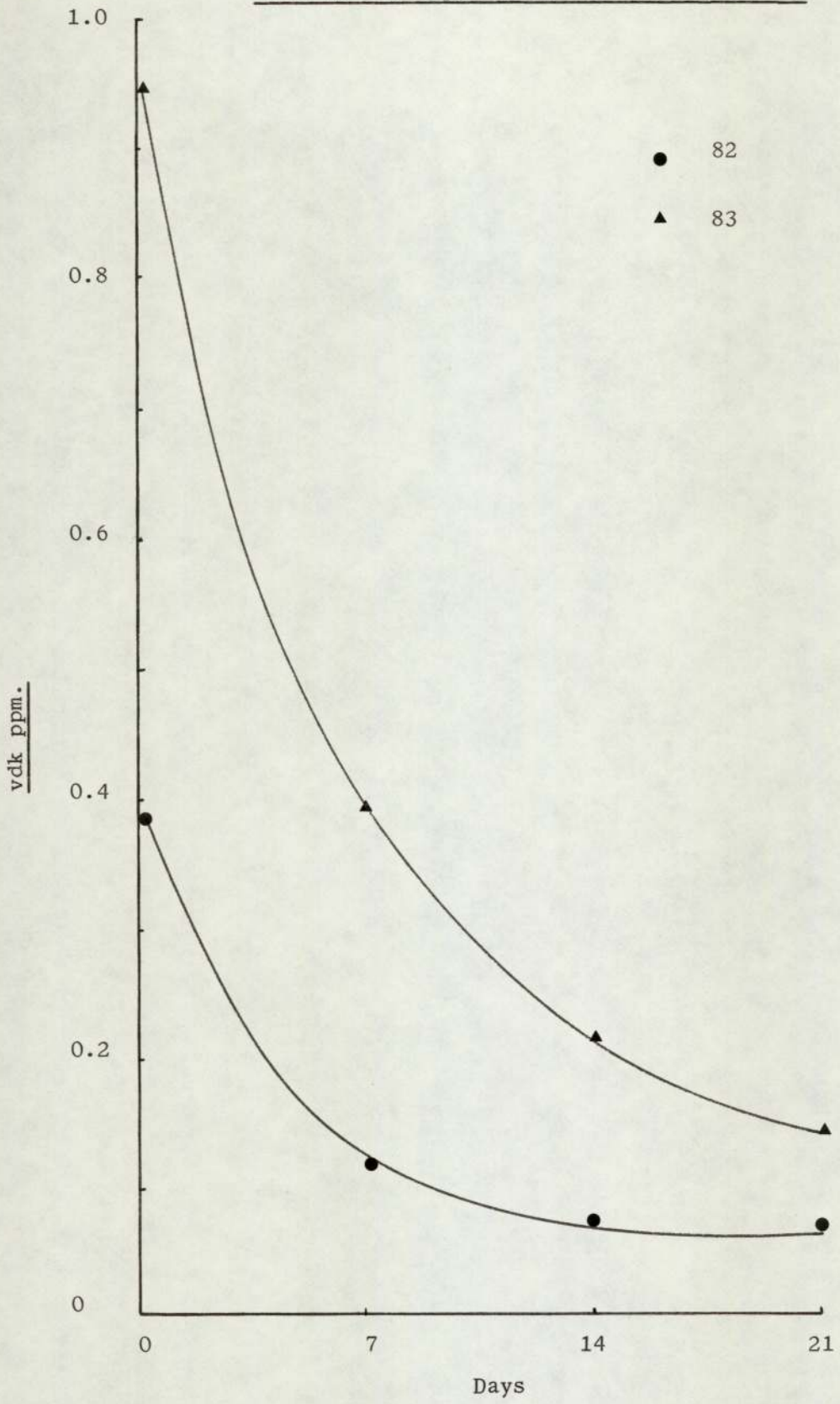
Graph 8

The change in levels of vicinal diketones with time in batch produced beer stored at 4°C



Graph 9

The change in levels of vicinal diketones with time in batch produced beer stored at $4^{o}C$



During storage at 4°C the amounts of vicinal diketones decreased. The rate and extent of the reduction was greatest in those beers which contained a high initial concentration of the compounds. (See Graphs 5 - 9 and Table 3.)

Table 3.

The changes in concentration of vicinal diketones in beers during storage at 4°C.

Beer No.	Initial conc. vdk. ppm.	Conc. vdk(ppm) at Day 21.	% Reduction
39	1.31	0.050	96.2
49	0.700	0.040	94.3
50	0.750	0.030	96.0
51	0.280	0.100	64.3
52	0.220	0.125	43.2
65	0.105	0.120	- 14.3
73	0.270	0.095	64.8
75	0.260	0.110	57.7
76	0.125	0.072	42.5
77	0.355	0.065	81.7
79	0.820	0.100	87.8
80	0.640	0.095	85.1
82	0.375	0.070	81.4
83	0.945	0.145	84.6

Secondary Selection

The yeasts used in the second stage of this investigation were selected after assessment against the following criteria:

i. The level of vdk. in the beer after 21 days storage at 4°C should not exceed 0.160 mg. per litre.

ii. The yeasts should gain a Modified Burns number greater than 35 when tested for flocculence by Sharp's method. Previous experience with strains of Saccharomyces cerevisiae indicated that yeasts which gained lower modified Burns numbers were washed out of the tower fermenter even at low volumetric efficiencies.

The following yeasts were selected:

Culture number	vdk in beer mg/litre	Modified Burns number
CFCC 39	0.050	78
CFCC 73	0.095	94
CFCC 75	0.110	46
CFCC 83	0.145	40

SECONDARY SCREENING

The second stage of the screening procedure involved the use of a laboratory tower fermenter.

Experiments were conducted to investigate the following:

i. The limits of the performance of the yeast in the laboratory tower fermenter.

ii. The levels of vicinal diketones in the beer at the limiting volumetric efficiency.

MATERIALS AND METHODS

i. Preparation of the tower fermenter

In early experiments the tower fermenter was sterilized by exposure to live steam for a minimum of 20 hours. In later experiments the fermenter was exposed to formalin vapour for 10 - 12 hours and then the vapour was displaced with live steam.

All fitting such as the wort lines, and air lines, were autoclaved at 1.05 kg/cm^2 for 15 minutes.

ii. Preparation of the wort

A suitable quantity of malt extract syrup was diluted with hot water to give a wort of the desired original gravity. The coagulated proteinaceous matter was allowed to settle, and the clear wort decanted into a 20 litre glass aspirator and autoclaved at 1.05 kg/cm^2 for 15 minutes. The heating cycle lasted a total of 1.25 hours. The sterile wort was allowed to cool to room temperature (18° to 23°C).

iii. Attenuation Limit

The attenuation limit of 1.050 wort was determined by conducting batch fermentations with yeast CFCC 83. Sterile wort (750 ml. in 1 litre Erlenmeyer flasks, plugged with cotton wool) was inoculated with 11.25 g. of yeast (centrifuged wet weight) and allowed to ferment without shaking for seven days. The temperature was controlled at 20°C.

Analyses of the wort and beer are given below:

	Specific Gravity	Total Reducing Sugar
Wort	1.050	7.08 g per 100 ml.
Beer	1.0122	1.38 g per 100 ml.

The reducing sugars were determined by the method of Lane and Eynon (1923).

iv. Operation of the tower fermenter

The tower fermenter was partially filled with wort and then inoculated with yeast which had been propagated in static culture at 30°C. The flow rate of the wort was adjusted to give a volumetric efficiency of approximately 0.5 during the first two days. The throughput was increased gradually thereafter to maintain the growth rate and the yeast usually grew to fill the fermenter in 10 - 14 days. Sterile air was pumped into the base of the fermenter during these early stages to promote the growth of the yeast. When the yeast had grown sufficiently the air supply was stopped. Thereafter the only oxygen introduced to the fermenter was that dissolved in the wort.

v. Analysis

The following analyses were performed routinely:

a. Present gravity of the beer
b. pH of the beer
c. Total acidity
d. Total vicinal diketones

) For details of the methods see Pages 76 and 77.

iv. Storage of the beer

Beer produced by tower fermentation was transferred through sterile lines to vertical stainless steel containers (9.8 litre capacity) supplied by A.P.V. Company Limited. The internal diameter of these vessels was 10.0 cm and the height 125 cm. The sample tube extended from the top of the container to 2.5 cm from the bottom. The vent tube was flush with the interior face of the top plate. Both tubes were fitted with rubber hosing externally.

These containers were prepared by steaming under slight pressure (up to 0.35 kg/cm^2) for 2 hours. It was possible to completely close these containers, but to prevent an excessive build up of pressure during secondary fermentation a bunsen valve was incorporated. Sampling was achieved by applying a top pressure with CO_2 gas.

RESULTS

i. Standard conditions

The tower fermenter was operated at 20°C with a wort of original gravity 1.050, and the performance of each yeast was assessed under these conditions.

ii. CFCC 39

a. Morphology

The morphology of this yeast depended upon the rate at which the fermenter was operated. At a volumetric efficiency of 1.6 the yeast formed fine flocs 0.1 -0.2 cm. diameter in the 2.54 cm section, but a plug of yeast was present at the base of the fermenter. By visual examination it was apparent that the yeast concentration decreased with distance from the base. At a volumetric efficiency of 2.6 the yeast was completely dispersed and the effluent beer contained a high concentration of yeast (3.69×10^8 cells/ml) (See Methods, Page 77,).

b. Limiting volumetric efficiency

The performance of the fermenter was limited by incomplete fermentation of the wort at speeds of operation in excess of volumetric efficiency 2.2. Simultaneously the yeast deflocculated and as a result the concentration of yeast in the beer leaving the fermenter increased from the level of 4.75×10^7 cells/ml at volumetric efficiency 1.8 to 3.69×10^8 cells/ml at volumetric efficiency 2.2.

The analysis of the beers produced at two volumetric efficiencies - one below and one above the limiting rate of operation are given below.

c. Analysis of the beer

The analysis of the beer produced at volumetric efficiency 1.8 is given below:

Wort O.G.	1.0495
Beer P.G.	1.010
pH (beer)	4.05
Total acidity (as acetic acid)	0.22 g per 100 ml
Total vdk	0.320 mg. per 100 ml.

The beer produced at volumetric efficiency 2.3 gave the following analysis:

Wort O.G.	1.049
Beer P.G.	1.0125
pH (beer)	4.25
Total acidity (as acetic acid)	0.28 g fer 100 ml
Total vdk	0.385 mg. per 100 ml.

d. Comment

The attenuation gravity of the beer (1.010) produced at volumetric efficiency 1.8 was lower than that of the beer produced by batch fermentation (1.0122) of a wort of similar specific gravity (see Materials and Methods). This phenomenon has been reported previously (Klopper et alia, 1965) and is known to be the result of the limited yeast growth which occurs when a tower fermenter is operated under almost totally anaerobic conditions. If the wort is aerated prior to entry into the base of the fermenter then the specific gravity of the beer from the tower fermenter is similar to that of beer produced by conventional batch fermentation. (Ault et alia, 1969).

The tower fermented beer was normal with respect to pH and total acidity, but the level of vicinal diketones was approximately three times greater than that found in matured lager beers.

The beer produced at volumetric efficiency 2.3 was incompletely fermented and the specific gravity (1.0125) was higher than the normal (1.009 - 1.011) for the tower fermented beer produced from this batch of malt extract. The pH of the beer was within the normal range (pH 4.00 - 4.30) for tower fermented beer.

iii. CFCC 73

a. Morphology

At rates of operation less than volumetric efficiency 1.0 this yeast formed large dense flocs which aggregated into a solid relatively impervious mass. As a result large pockets of gas and liquid were trapped between plugs of yeast in the 2.54 cm section of the fermenter. Progressive increases in the rate of operation caused progressive deflocculation of the yeast upwards from the base of the tower until at volumetric efficiency 2.0 the yeast was completely dispersed in the form of small flocs less than 0.1 cm diameter.

b. Limiting flow rate

At volumetric efficiencies greater than 1.6 the present gravity of the effluent beer began to rise indicating that fermentation was incomplete.

At a volumetric efficiency 2.0 the yeast was completely dispersed as small flocs and any increase above this rate caused substantial washout of yeast.

c. Analysis of the beer

The analysis for the beer produced at volumetric efficiency 1.5 is given below:

Wort O.G.	1.0485
Beer P.G.	1.0115
pH (beer)	4.1
Total acidity (as acetic acid)	1.02 g per 100 ml
Total v.d.k.	0.730 mg per 100 ml

d. Comment

The beer produced by this yeast contained a high concentration of vicinal diketones. The total acidity was also abnormally high. Both of these conditions may occur if bacteria of the genus Lactobacillus or Pediococcus gain access to the tower fermenter though microscopic examination did not reveal any of these micro-organisms.

iv. CFCC 75

a. Morphology

This yeast gained a modified Burn's number of 46 by Sharp's test. In the tower fermenter flocs of approximately 0.1 cm diameter formed, and in consequence the yeast was dispersed throughout the fermenter. The carbon dioxide produced by the yeast produced some longitudinal mixing of the contents of the fermenter.

b. Limiting flow rate

At a volumetric efficiency of 0.9 considerable washout of the yeast occurred. At this rate of operation the superficial liquid velocity in the 2.5 cm section was 3.8×10^{-2} cm. sec.

c. Analysis of the beer

Analysis of the beer produced at volumetric efficiency

0.5 gave the following results:

Wort O.G.	1.0485
Beer P.G.	1.0095
pH (beer)	4.45
Total acidity (as acetic acid)	0.26 g per 100 ml
Total vdk	0.325 mg per 100 ml

d. Comment

The pH and the vicinal diketone content of this beer were high.

v. CFCC 83

a. Morphology

A modified Burn's number of 39.8 was obtained for this yeast by using Sharp's test.

The behaviour of the yeast varied with the rate at which the fermenter was operated. At volumetric efficiencies less than 1.0 the yeast in the 2.54 cm. section of the fermenter formed fine flocs of approximately 0.1 - 0.2 cm. diameter and a plug of yeast was present at the base. The yeast in the 7.5 cm. diameter section flocculated to form a loose plug from which large lumps were periodically detached by the action of fermentation gas. The lumps of yeast then fell to the base of the fermenter and in this way the 2.54 cm. section became packed with yeast, which then slowly deflocculated and another cycle of these events commenced.

Above a volumetric efficiency of 1.0 the yeast dispersed to form small flocs which were present in all regions of the fermenter.

b. Limiting flow rate

The performance of the fermenter was satisfactory at all

rates of operation up to volumetric efficiency 2.3 when a slight rise in the specific gravity of the beer was noted.

c. Analysis of the beer

Analysis of the beer produced at a volumetric efficiency of 0.5 gave the following results:

Wort O.G.	1.049
Beer P.G.	1.0117
pH (beer)	3.98
Total acidity (as acetic acid)	0.25 g per 100 ml
Total vdk	0.110 mg per 1000 ml

At a volumetric efficiency of 2.3 the beer had the following composition:

Wort O.G.	1.0497
Beer P.G.	1.0122
ph (beer)	3.9
Total acidity (as acetic acid)	0.25 g per 100 ml
Total vdk	0.543 mg per 1000 ml

Discussion and conclusions

When using yeast CFCC 73 and CFCC 75 the performance of the fermenter was limited by the physical washout of the yeast. In the case of CFCC 75 it was impossible to establish a high concentration of yeast within the fermenter because of the very fine flocs washed out of the fermenter at all rates of operation. The morphology of yeast CFCC 73 altered with the rate at which the fermenter was operated. At low rates the yeast behaved as a flocculent yeast but with increasing flow rates the morphology changed to that typical of a non-flocculent yeast. This alteration in morphology was presumably in response to the presence of unfermented sugar in the wort, since the beer produced at volumetric efficiencies greater than 1.6 was not completely fermented.

The behaviour of yeast CFCC 83 was similar to that of yeast CFCC 73 but when the yeast dispersed at high rates of operation no serious washout of the yeast occurred because the flocs were of sufficient size to sediment out of the beer at the head of the fermenter. The performance of the fermenter was limited by incomplete fermentation of the wort at volumetric efficiencies greater than 2.3.

Yeast CFCC 39 was the best of the four cultures examined. The bed of yeast in the 2.5 cm. section of the fermenter did not disperse until the fermenter was operated at a volumetric efficiency of 2.6 but the performance of the fermenter was limited by incomplete fermentation of the wort at volumetric efficiencies greater than 2.2.

The yeast CFCC 75 may be regarded as non-flocculent in the classification of Greenshields and Smith,(1971). The yeasts CFCC 39, CFCC 73, and CFCC 83 may be considered to fit into the class of flocculent - fermentation limited yeasts. However, the culture CFCC 39 was unusual in the sense that washout of the yeast occurred at the rate at which incomplete fermentation of the wort was first apparent.

It appears that the yeast CFCC 39 will deflocculate under the influence of low concentrations of fermentable sugar. Thus to avoid washout it is essential to operate the fermenter at a speed which permits complete fermentation of the wort.

This investigation indicated that yeast CFCC 75 was not sufficiently flocculent for use in the tower fermenter. Yeasts CFCC 39, 73 and 83 were considered to perform satisfactorily in the tower fermenter though yeast CFCC 73 was shown to produce a beer with a high acid and vicinal diketone content. Therefore yeasts CFCC 39 and 83 were selected as the cultures to be used for investigating the influence of the operational parameters upon the vicinal diketone levels in beer from the tower fermenter.

vi. Storage of the tower beer

a. Storage at 4°C

The beers produced by tower fermentation with the yeasts CFCC 39, CFCC 73 and CFCC 83 were stored at 4°C, and the levels of vicinal diketones estimated at intervals.

The results have been presented on Graphs 10 - 12.

It is apparent that in all cases the reduction in the level of vicinal diketones was slower than was observed in the batch beer produced by the same yeasts. The content of vicinal diketones fell to approximately 30% of the initial level in between 14 and 21 days, but thereafter little further change occured. After 28 days storage the beers produced by yeasts CFCC 39 and CFCC 73 still contained more than 0.20 ppm. of vicinal diketones . The pattern of the changes occuring in the beer from yeast CFCC 83 was similar except the gradual reduction continued throughout the period of storage (Graph 12).

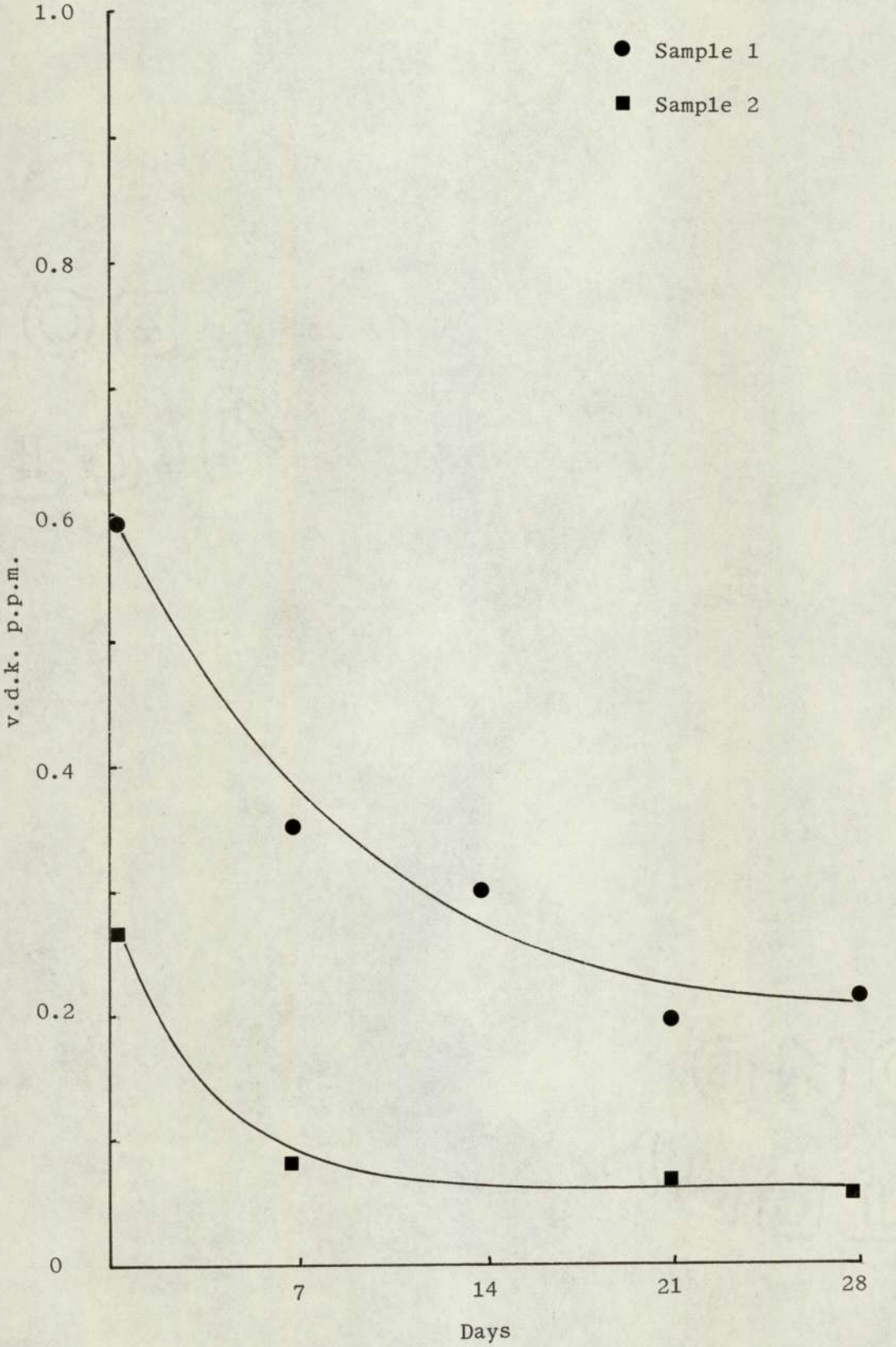
b. Comparison of storage at 4°C and 21°C

Because the rate of fall in the levels of vicinal diketones in tower fermented beer was slow at 4°C, the effect of storage at a higher temperature was investigated.

The beer produced on one day by yeast CFCC 39 was collected into two stainless steel vessels. The vessels were 10 cm in diameter and 125 cm in length. Two stainless steel tubes passed through the top end cap which screwed down onto a sealing washer. One of the tubes terminated level with the inside face of the top end cap, but the other passed down the vessel to within 2.5 cm of the base. The total volume of each vessel was 9.8 litres. Sampling

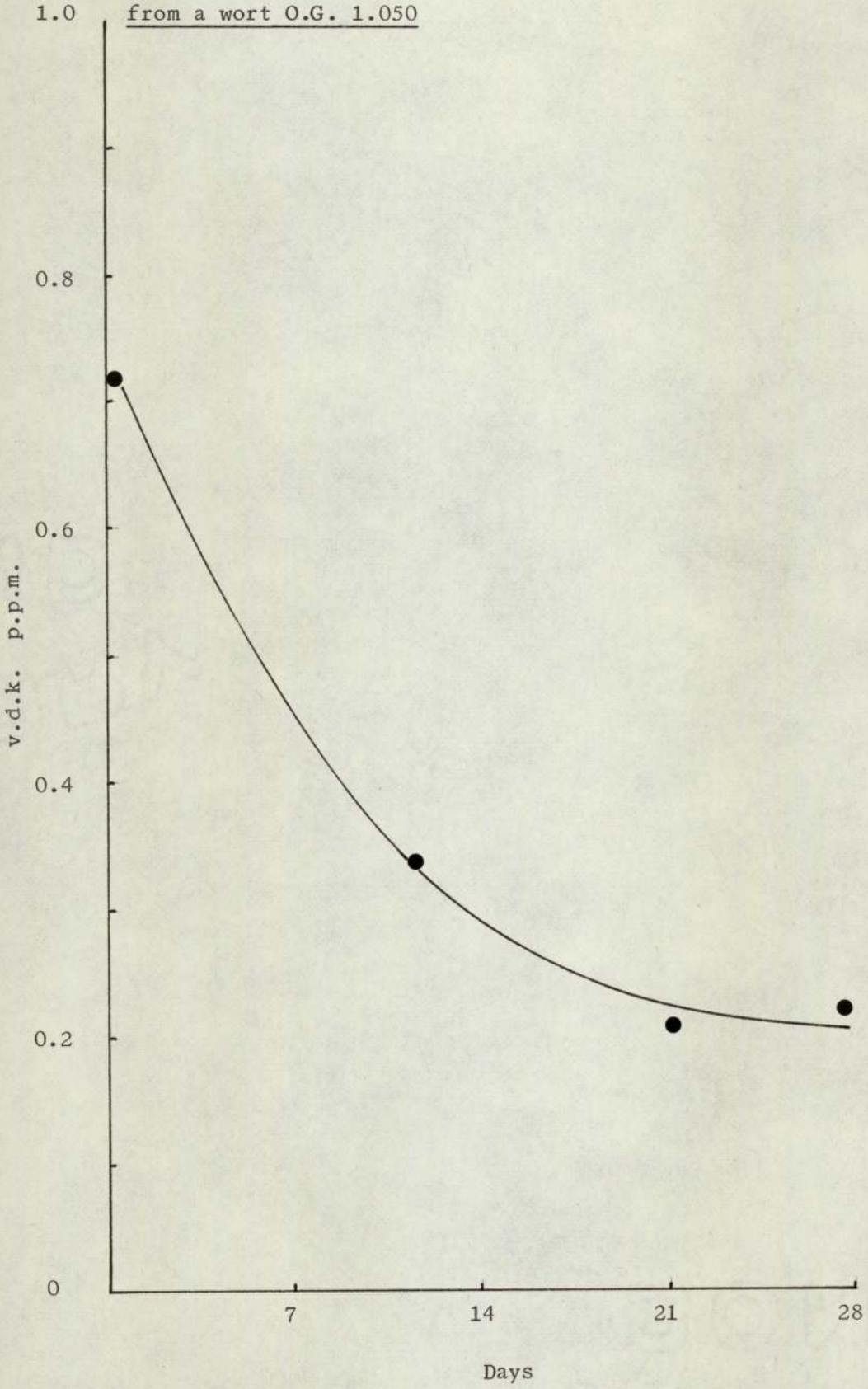
Graph 10

Changes in the vicinal diketone content of tower beer during storage at 4°C. Beer produced by yeast CFCC 39 at 20°C from a wort O.G. 1.050



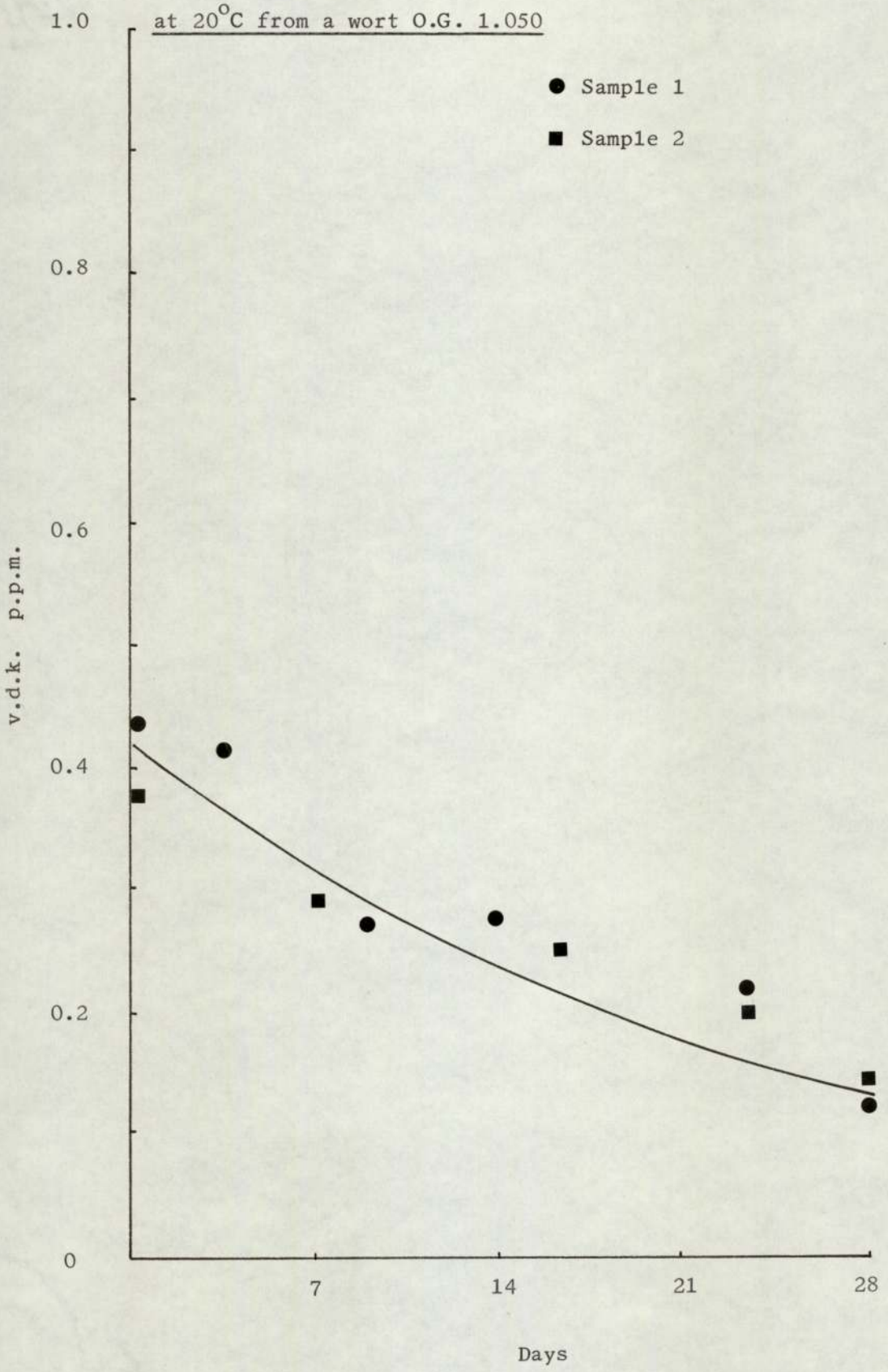
Graph 11

Changes in the vicinal diketone content of tower beer during storage at 4°C. Beer produced by CFCC 73 at 20°C from a wort O.G. 1.050



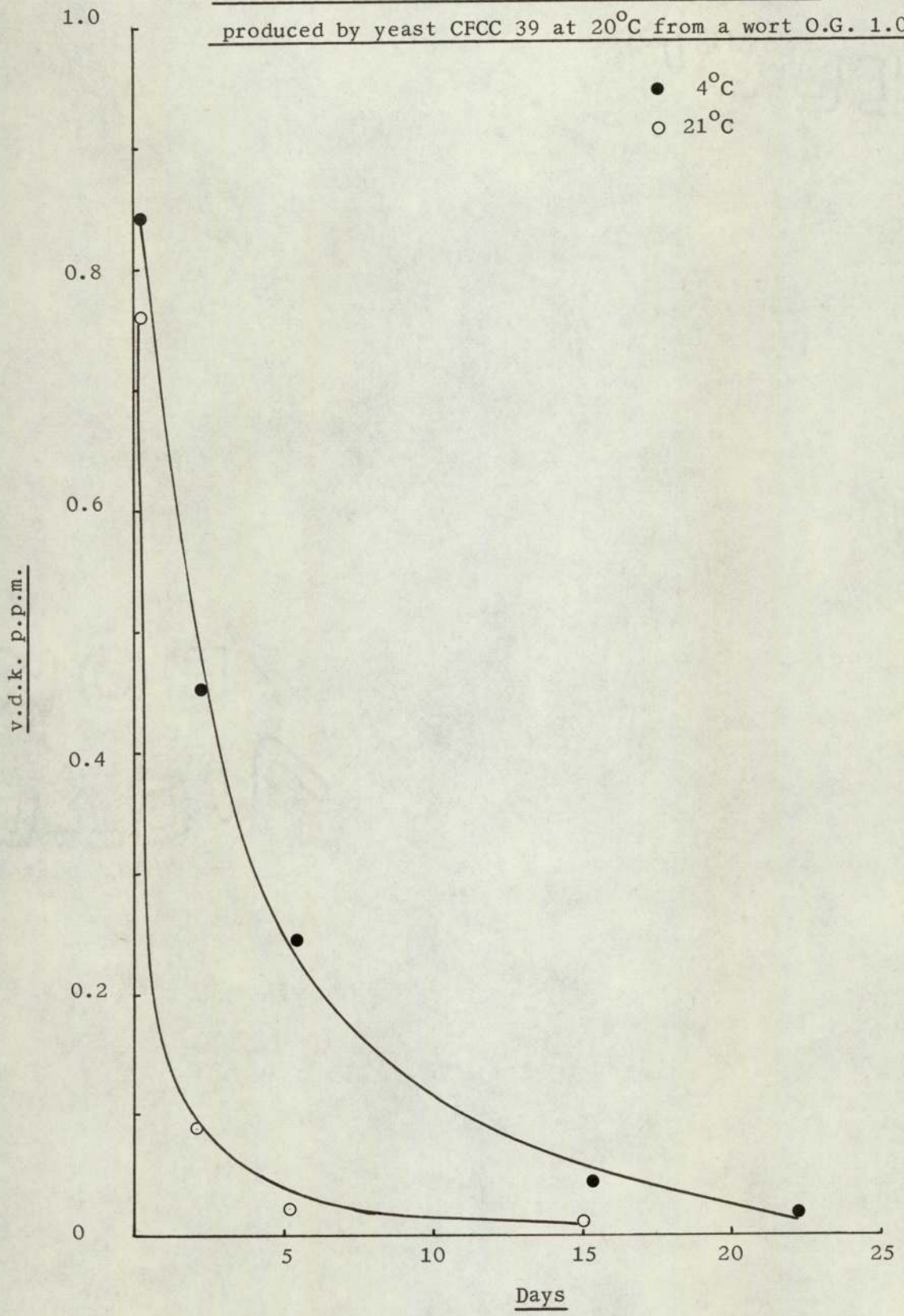
Graph 12

Changes in the vicinal diketone content of tower beer during storage at 4°C. Beer produced by yeast CFCC 83 at 20°C from a wort O.G. 1.050



Graph 13

The effect of storage temperature upon the rate of reduction in levels of vicinal diketones in beer produced by yeast CFCC 39 at 20°C from a wort O.G. 1.055



was achieved by applying top pressure with carbon dioxide.

The results recorded on Graph 13 show that the level of vicinal diketones fell to 0.1 ppm in between 1 and 2 days at 21°C, whereas a similar level was reached after 10 days at 4°C. This finding is in agreement with previous investigations (den Blanken, 1971; Linko and Enari, 1967; Kraus and Sommer, 1967).

Conclusions

A satisfactory and rapid reduction in the concentrations of vicinal diketones in tower produced beer may be achieved if the beer is maintained at a temperature of 21°C for a short period at the commencement of the lagering process.

Summary

During a storage at 4°C the level of vicinal diketones in the tower produced beer fell gradually until approximately 33% of the initial level was reached. A more rapid reduction in the levels of vicinal diketones occurred when the beer was stored at 21°C.

INVESTIGATION OF THE FACTORS INFLUENCING THE VICINAL DIKETONE CONTENT OF TOWER BEER

Experiments were conducted with the yeasts CFCC 39 and CFCC 83 in order to establish the effect of alterations in the following operational parameters:

i. Wort flow rate.

ii. Aeration

iii. Temperature

iv. Wort strength

MATERIALS AND METHODS

The following analyses were carried out:

i. Specific gravity - of the wort and beer.

ii. pH

iii. Total acidity

iv. Total vicinal diketones

v. Yeast count

The methods by which the above were performed have been detailed previously.

RESULTS

i. Wort flow rate

The results for individual samples have been recorded in the Appendix (Page xi).

The relationship between the levels of vicinal diketones in beer and the wort flow rate was established with the aid of statistical analysis. The method of least squares was used to obtain the best straight line relationship and the coefficient correlation.

a. CFCC 39

An experiment was performed to determine the effect of wort flow rate upon the levels of vicinal diketones in the beer.

Experimental conditions	
Wort original gravity	1.050
Temperature	$20^{o}C$

The results recorded in the Appendix (Page xi) indicated that fluctuations in the level of vicinal diketones in beer occurred even when the wort flow rate was held steady. However, a definite trend towards increased levels of vicinal diketones in beers produced at higher wort flow rates was evident (see Graph 14).

The equation of the line and the coefficient of correlation (r) are given below:

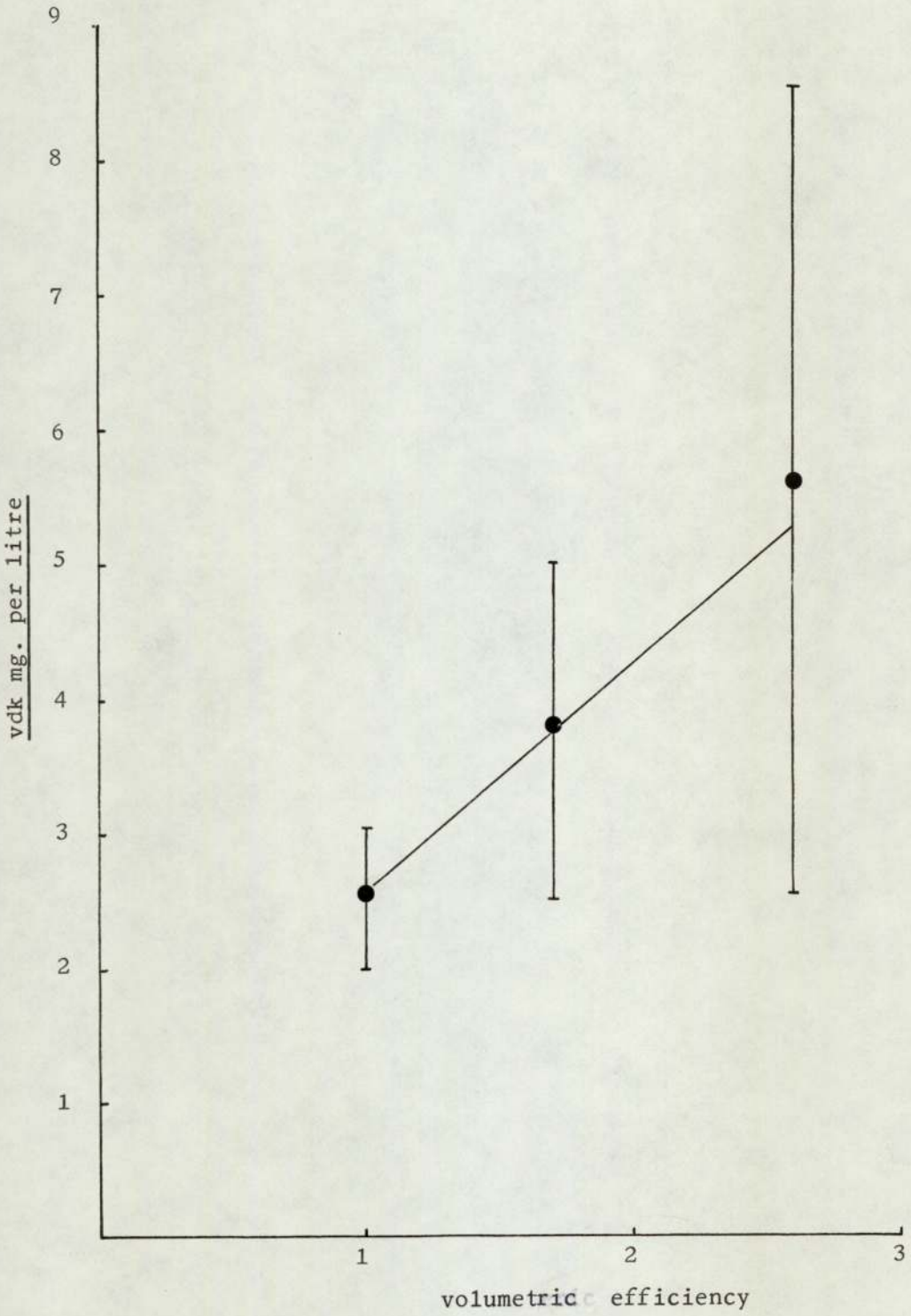
Equation of the line $y = 0.164x + 0.093$

Coefficient of correlation (r) = 0.803

Included on the graph are the mean values and the 95% ranges for the levels of vicinal diketones in the beer produced at various volumetric efficiencies.

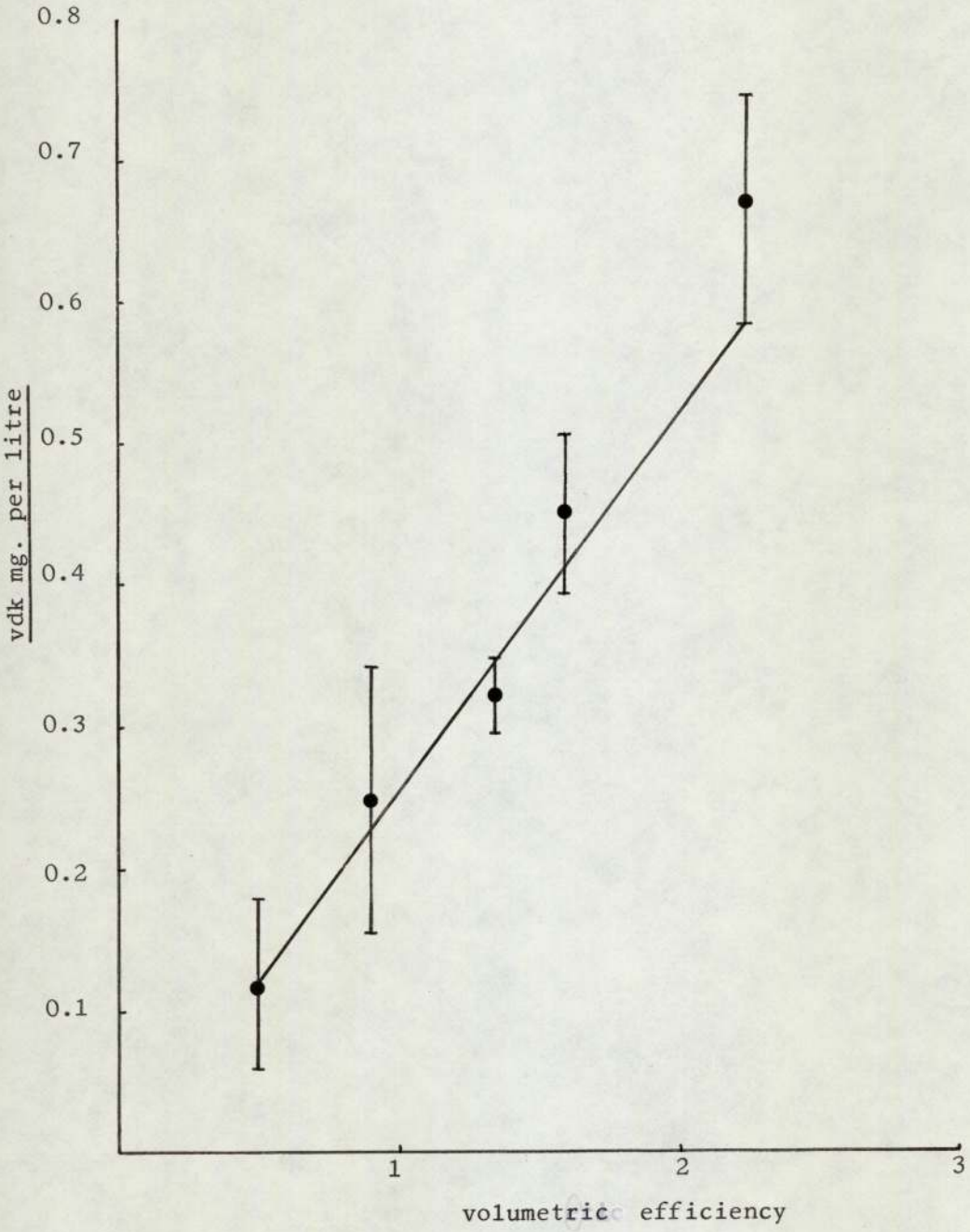
Graph 14

Relationship between vicinal diketones in beer and volumetric efficiency with yeast CFCC 39 fermenting wort O.G. 1.050 at 20°C.



Graph 15.

Relationship between vicinal diketones in beer and volumetric efficiency with yeast CFCC 83 fermenting wort O.G. 1.050 at 20°C.



b. CFCC 83

A similar experiment was conducted with yeast CFCC 83.

Experimental conditions

Wort original gravity	1.050
Temperature	20°C

The details of individual samples have been recorded in the Appendix.

The best straight line has been plotted on Graph 15.

Equation of the line $y = 0.257x + 0.010$

Coefficient of correlation (r) = 0.964

The relationship of vicinal diketones to the wort flow rate was similar to that observed with yeast CFCC 39.

c. CFCC 83

A second experiment was conducted with yeast CFCC 83 to determine the relationship between wort flow rate and vicinal diketones in the beer at a different temperature.

Experimental conditions

Wort original gravity	1.050
Temperature	15°C

The detail of individual samples have been recorded in the Appendix.

The result of analysis has been presented on Graph 16. A similar relationship exists to that observed when using this yeast at a temperature of 20°C.

Equation of the line $y = 0.302x + 0.068$

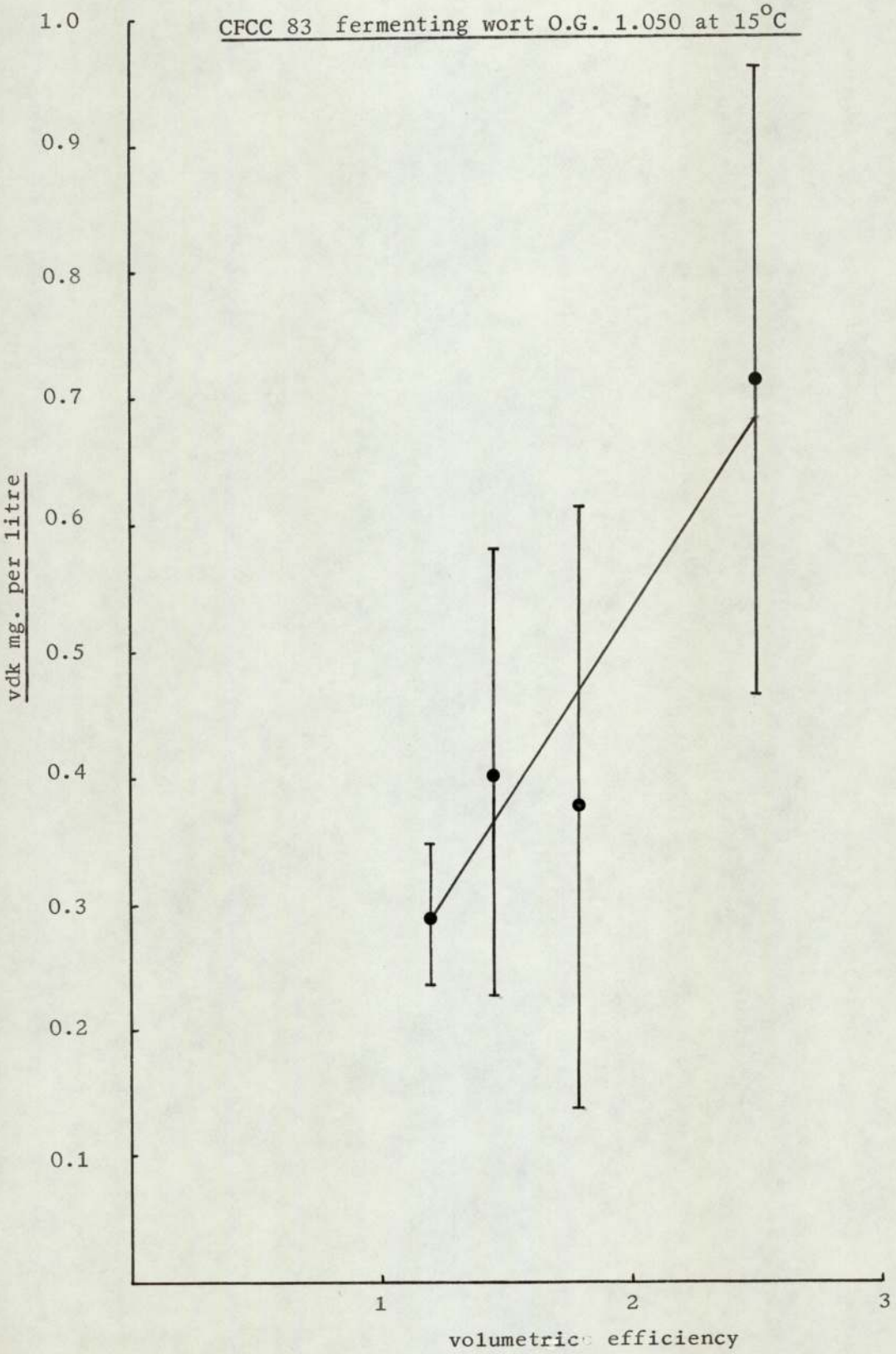
Coefficient of correlation(r) = 0.803

Discussion and conclusions

The results of this investigation show that the level of vicinal diketones in a beer leaving the fermenter depends

Graph 16

Relationship between vicinal diketones in beer and volumetric efficiency with yeast CFCC 83 fermenting wort O.G. 1.050 at 15°C



to a large extent upon the rate of operation of the fermenter. At high wort flow rates the concentration of vicinal diketones in the beer is generally high but may fluctuate within a wide range of values. Statistical analysis indicated that in some cases at constant flow rates the highest level to be expected may be four times greater than the lowest level. The gross fluctuations in the levels of these compounds may be due to longitudinal mixing within the fermenter, and to ensure that the beer contains less than 0.200 ppm of vicinal diketones it is necessary to operate the fermenter at a volumetric efficiency less than 1.0.

ii. Aeration

The influence of aeration upon the levels of vicinal diketones in the beer was studied by operating the fermenter at a steady rate for a period of five days with aeration and for a similar period without aeration.

Experimental conditions

Yeast	CFCC 39
Wort original gravity	1.050
Fermenter rate (E)	1.7
Temperature	20°C
Aeration rate	120 ml. per min.

The results have been presented in Table 4. overleaf.

Table 4.

The effect of aeration upon levels of v.d.k. in tower fermented beer

Day	Aeration	vdk.(p.p.m.)	pH (beer)	Beer P.G.
1	+	0.885	4.4	1.0125
2	+	0.615	4.4	1.0107
3	+	0.680	4.0	1.0105
4	+	0.930	3.9	1.0112
4	+	0.915	3.95	1.0107
5	+	0.765	4.1	1.0105
14	-	0.330	4.2	1.0112
15	-	0.490	4.2	1.0113
16	-	0.330	4.2	1.0110
17	-	0.380	4.15	1.0115
18	-	0.400	4.3	1.0115
19	-	0.32	4.05	1.010

Analysis of the results gave the following:

	Aerated	Non-aerated
Range of values	0.615 - 0.930	0.320 - 0.490
Standard deviation	0.132	0.065
Variance	0.175	0.0042

Application of the 'F' test indicated that the results of the students 't' test were valid.

$F = 4.2$

degrees of freedom = $n_1 = 5$ $n_2 = 5$

Students 't' test was applied to analyse the differences between the two groups of results. The difference between the

treatments was highly significant.

$$t = 7.752$$

$$p = 0.001$$

Conclusion

It was conclusively shown that direct injection of air into the base of the fermenter caused an increase in the concentration of vicinal diketones in the beer.

Discussion

It is known that if aeration is applied towards the end of a batch fermentation the resulting beer may contain an unacceptable concentration of vicinal diketones. This is probably due to yeast growth during which α-acetolactate is formed. This substance decomposes only slowly in beer at low temperatures and in consequence diacetyl and 2.3-pentanedione arise in beer after yeast activity has ceased.

In the laboratory tower fermenter the high levels of vicinal diketones in the beer was probably caused by mixing of the fermenter contents and some yeast growth at the head of the tower. It is possible that the fluctuations in the present gravity and pH of the beer reflect a partial breakdown of the gradient within the fermenter. In this context it is noteworthy that when the fermenter was not aerated the pH and present gravity of the beer were more stable.

It has been shown by Ault, et alia (1969) that some aeration of the fermenter is essential to maintain yeast viability and control the production of esters. From the results of this study direct aeration is contra-indicated but aeration of the wort prior to entry into the fermenter would overcome this problem.

iii. Temperature

a. Comparison of the two temperatures

The influence of temperature upon the concentration of vicinal diketones in the beer was investigated. The fermenter was operated at a steady rate and readings taken on four consecutive days at one temperature and then for a similar period at a second temperature.

Experimental conditions

Wort original gravity	1.050
Wort flow rate (E)	1.3
Aeration	-

The results have been presented in Table 5.

Table 5.

Effect of temperature upon the concentration of vicinal diketones in tower fermented beer.

Day	Temp. ^{o}C	vdk. ppm	pH (beer)	Beer P.G.
1	20	0.330	3.8	1.0125
2	20	0.325	3.95	1.0123
3	20	0.430	3.85	1.0132
4	20	0.492	3.80	1.0130
8	15	0.325	3.75	1.0122
9	15	0.290	3.85	1.0120
10	15	0.420	3.80	1.0122
11	15	0.500	-	1.0127

The mean values for the concentration of vicinal diketones are:

$20^{o}C$	0.469 ppm
$15^{o}C$	0.444 ppm

It is evident without further analysis that there is no substantial difference between the concentrations of vicinal diketones in the beer produced at 15°C and 20°C.

b. Comparison of three temperatures

A second experiment was conducted at a lower rate of operation and the levels of vicinal diketones in beer produced at three different temperatures were investigated.

Experimental conditions

Wort original gravity	1.050
Wort flow rate (E)	0.5
Aeration	-

The fermenter was operated for three consecutive days at each temperature. The results are presented in Graph and Table 6.

Table 6.

The effect of temperature upon the level of vicinal diketones in tower fermented beer

Day	Temp. °C	vdk. (ppm)	pH (beer)	Beer P.G.
1	15	0.395	3.50	1.0117
2	15	0.450	3.73	1.0119
3	15	0.500	3.80	1.0121
4	20	0.365	4.17	1.0115
5	20	0.440	4.20	1.0118
6	20	0.370	4.18	1.0116
7	27	0.195	4.20	1.0112
8	27	0.090	4.20	1.0113
9	27	0.120	4.21	1.0114

The mean values for the level of vicinal diketones are given below:

Temp. °C	vdk (ppm)
15	0.446
20	0.391
27	0.133

c. Discussion

The results of both experiments indicate that there is little difference between the concentrations of vicinal diketones in beer produced at 15°C and at 20°C. However a substantial reduction in the levels of vicinal diketones was achieved by operating the fermenter at 27°C. At this temperature the yeast deflocculated and the yeast mass was depleted by washout.

The following figures relating to the yeast concentration in the beer leaving the fermenter serve to illustrate the degree of washout which occured.

Temp. °C	Yeast count (cells per ml.)
15	$0.28 - 1.6 \times 10^6$
20	$0.31 - 1.94 \times 10^6$
27	19.0×10^6

iv. Wort strength

The results of experiments conducted under similar conditions though not necessarily at the same time have been analysed and the results summarized below.

a. Operating the fermenter at a steady flow rate

Experimental conditions

Yeast	CFCC 39
Temperature	20°C
Wort flow rate (E)	1.0

Table 7.

The effect of wort original gravity upon the levels of vicinal diketones in tower fermented beer.

Wort O. G.	Beer P.G.	vdk. (ppm)	pH (beer)
1.040	1.0088	0.185	4.4
1.040	1.0087	0.185	4.35
1.045	1.0093	0.350	4.55
1.045	1.0090	0.350	4.5
1.050	1.0105	0.225	-
1.050	1.0113	0.260	-
1.050	1.0097	0.275	-

By examination of these values it is apparent that wort gravities in the range normally used for brewing have little effect upon the level of vicinal diketones in the beer.

The influence of wort gravity upon the rate at which a tower fermenter can be operated has been established by Greenshields and Smith (1971), and the relationship was confirmed for a second yeast (see Page 37). With regard to these findings the results of experiments were examined by another approach.

b. Operating the fermenter at the limiting flow rate

An analysis was made of the results obtained when the fermenter was operated at the limiting flow rate with normal and high gravity worts.

Experimental conditions

Yeast	CFCC 83
Temperature	20°C

Table 8.

Comparison of the vicinal diketones in beer from worts of various original gravities

Wort O. G.	Wort flow rate (E)	vdk. (ppm)	Beer P. G.	pH (beer)
1.050	2.3	0.600	1.0127	4.2
		0.543	1.013	3.8
		0.492	1.013	3.85
1.060	1.5	0.300	1.0140	3.9
		0.210	1.0142	3.8
1.080	0.36	0.180	1.0227	4.20
		0.190	1.0237	4.17
		0.250	1.0248	4.20

From the results it is evident that when operating the fermenter at the limiting flow rate the beer produced from a wort of original gravity 1.080 contained less vicinal diketones than that from a wort of original gravity 1.050.

c. Discussion

The above analyses suggested that the time span of the fermentation may bear more influence upon the level of vicinal diketones than the original gravity of the wort. The results of operating the fermenter at similar flow rates with two worts are presented overleaf and

seem to confirm this explanation.

Table 9.

Comparison of the vicinal diketones in beers from worts of different original gravities produced at a low volumetric efficiency.

Wort O. G.	Temperature °C	Volumeteric Efficiency	vdk. mg. per litre	Beer P. G.
1.050	20	0.43	0.108	1.0122
			0.107	1.0125
			0.110	1.0117
1.080	20	0.36	0.180	1.0227
			0.250	1.0243
			0.190	1.0237

v. General Discussion and Conclusions

The results of the experiments upon the effect of wort flow rate and temperature indicated that beer containing an acceptable level of vicinal diketones may be obtained from the tower fermenter at a low volumetric efficiency. The use of elevated temperatures is also beneficial. At first glance this finding is in conflict with Portno's report (1966a) that the use of high temperatures and yeast concentrations caused an increase in the production of vicinal diketones in batch fermenter beer. However, Liebs et alia, (1970) reported that the use of similar conditions during the lagering period can bring about a rapid reduction in the vicinal diketone content of beer. Wainwright (1973) has stressed the fact that the use of certain methods, including that of the Institute of Brewing (Ault, 1968), can lead to erroneously high results for the level of vicinal diketones in beer due to spontaneous

decomposition of acetohydroxy acids during the distillation procedure.

It has been established that during fermentation α-acetolactate accumulates in the wort when the oxidation-reduction potential drops below rH 10. Inoue, et alia (1968a) reported that under anaerobic conditions at 20°C only 13% of α-acetolactate was converted to diacetyl after 6 hours.

The high concentration of yeast within a tower fermenter will ensure that the wort is anaerobic. This, together with the comparitively low residence times of the wort (10.5 hours at the highest flow rate used in this investigation) in a tower fermenter, suggests that in all probability comparitively large amounts of acetohydroxy acids are present in the beer leaving the fermenter. It seems likely that any vicinal diketones which form within the tower fermenter are rapidly removed by the yeast and that the apparently high levels of vicinal diketones in tower beer are in fact due to the presence of unconverted acetohydroxy acids which subsequently decompose.

The conclusions to be drawn from these investigations are:-

a. Any oxygen introduced into the fermenter should be dissolved in the wort and no gaseous oxygen should be present.

b. The fermenter should be operated at the highest temperature compatible with production of a beer with an acceptable flavour.

c. To obtain a beer containing a low level of vicinal diketones it is necessary to operate the fermenter at a low volumetric efficiency.

d. The yeast strain should be a flocculent type so that it will remain within the fermenter.

It is a simple matter to comply with the points listed above, but point c. carries serious implications with it. One of the major attractions to the brewer of the tower fermentation process is the rapid fermentation which can be achieved. If it is necessary to operate the fermenter at a very low volumetric efficiency then it is possible that the process will not be economic. Ault, et alia (1969) have shown that the tower fermenter can produce a beer over a range of flow rates. The lowest flow rate used by these workers corresponded to a wort residence time of 8 hours and it is interesting to note that the level of vicinal diketones in the beer produced at this flow rate were similar to those observed in beer from the laboratory tower fermenter when operating at the fastest rate - a residence time of 10.5 hours.

The elevated levels of vicinal diketones in tower fermented beer appear to be a consequence of the very short fermentation time, and it seems that it will be necessary to treat the beer after it leaves the tower fermenter. One method of achieving a rapid maturation of beer is to allow the secondary fermentation to proceed at a relatively high temperature. Recently Baker and Kirsop (1973) have reported that the beers containing high levels of vicinal diketones and their precursors may be rapidly treated by heating the beer to 60^{o}C for 15 minutes and then passing the beer through a vessel which contains a high concentration of yeast. By employing this method of post fermentation treatment it should be possible to realise the full potential of the tower fermenter.

SECTION 4

THE FERMENTATION OF BEET MOLASSES

A. THE SELECTION OF YEASTS BY A BATCH FERMENTATION TECHNIQUE

B. THE CONTINUOUS TOWER FERMENTATION OF MOLASSES

INTRODUCTION

One of the simplest fermentation processes is that in which carbohydrate substrates are fermented to ethanol for industrial purposes, and until the advent of the petrochemical industry all industrial alcohol was produced by fermentation.

The first half of the twentieth century saw a progressive decline in the production of alcohol from agricultural sources as the synthetic product became considerably cheaper to manufacture.

Although there is a steady demand for fermented alcohol in the food and pharmaceutical industry, other outlets for fermented alcohol have been suggested, and the use of fermented alcohol as a motor fuel was advocated many years ago (Hilbert, 1950). Recently the economics of alcohol production by synthetic and fermentation processes were re-examined by Miller (1971) who showed that alcohol from wheat was competitive economically with that synthesized from ethylene. The recent massive increases in the price of oil and the fact that there is virtually no surplus U.S. wheat have probably rendered Miller's estimates of 1971 inaccurate. To quote Miller (1973) ' The domestic and international situation for agricultural products has been so unstable recently that accurate predictions and planning have become practically impossible. ..... Possibly the past year would indicate that, in general, agriculture could contribute little additional to future United States industrial energy requirements. However, I do not believe the situation has so permenantly reversed'.

It seems probable that the present trends towards improved utilization of natural and agricultural resources may initiate a revival in the production of alcohol and other solvents by

fermentation. In addition the impending rules of the European Economic Community require that all potable alcohol and that for use in the pharmaceutical and cosmetic industries be derived from agricultural sources. It appears therefore, that fermentation will play an increasingly important role in the production of industrial alcohol.

There have been many attempts to produce industrial alcohol by continuous fermentation processes but to date there have been no reports of the application of the tower fermenter in such a system.

The media employed in the production of industrial alcohol consist of aqueous solutions of simple or complex sugars which can be converted to simple sugars. A wide range of raw materials are used but in general they may be classified into three groups:

i. The saccharine materials such as sugar cane, sugar beets, molasses, fruit juices and sulphite liquor.

ii. The starch materials including cereals such as wheat, barley, maize, oats, and root crops such as potatoes.

iii. The cellulosic materials such as wood.

The type of raw material in use varies from country to country depending upon availability and price. Thus in the U.S.A. grain is used extensively whereas in the United Kingdom cane molasses is the major raw material. In Germany and Poland potatoes serve as a major raw material, but in Sweden sulphite liquor from the paper industry is utilized.

The raw material used in this investigation was beet molasses a byproduct of the sugar refining industry. It is a dark brown viscous liquid containing 50 - 55% of fermentable sugar, mainly sucrose. Beet molasses has been used as a raw material for alcoholic fermentation in Europe and the Soviet Union but in

Britain the major uses are as cattle feed and in the production of baker's yeast, though it is also used in a mixture with cane molasses for alcohol production.

The methods by which the various raw materials are converted to alcohol and then distilled have been described in various texts (Prescott and Dunn, 1949; Hodge and Hildebrandt, 1954; Whitmarsh, 1958; Harrison and Graham, 1970) which should be consulted for details of industrial processes. The sugary materials such as molasses are normally diluted with water, salts added and the pH adjusted before pitching with yeast. The fermentations are usually completed in 48 - 72 hours depending upon the exact nature of the substrate employed.

The technology of the batch fermentation is simple and has been long established. Recent research has been directed towards:

i. Obtaining higher yields.

The Usines de Melle process (Boinot, 1939) represented a development of the batch process. In the conventional batch fermentation approximately 2 - 3% of the sugar is utilized in yeast growth, and is therefore not available for fermentation to alcohol. It was claimed that this loss was avoided by use of the Melle process in which the same yeast was employed for a number of fermentations. The yeast was recovered from the beer at the end of fermentation by centrifugation and the complete crop used to pitch the next fermentation cycle. Contamination problems were minimized by washing the yeast between recovery and reuse. Lagomasino (1949) claimed that formation of new yeast was almost completely avoided and alcohol yields as high as 97% of theoretical were

obtained. This process has been used successfully in France for the fermentation of blackstrap molasses and in the United States for sulphite liquor. Economic reasons have precluded the use of this process in Britain.

The use of molasses stillage as a substrate for yeast growth was advocated by Hildebrandt and Erb (1939a, 1939b). This development minimized the use of fresh molasses in the yeast production stage and had an additional advantage in reducing waste disposal costs.

ii. Developing a continuous fermentation process.

Interest in continuous fermentation techniques dates back to the last decade of the 19th century but many of the early attempts were directed towards the production of alcoholic beverages such as beer. Because of problems with contamination many of these early attempts failed, and interest in continuous fermentation lapsed.

It was Alzola (1945) who revived interest in the continuous fermentation techniques with a design for a multivessel system for the continuous fermentation of molasses. The system proposed by Victerero (1948) consisted of a series of tanks placed one upon the other to give the form of a tower in which the mash flowed from the base to the head. A laboratory scale single vessel system was described by Bilford et alia (1942) who claimed that the yield of alcohol was comparable to that obtained by conventional batch fermentation. Although continuous fermentations failed to become established in Britain or the U.S.A. this has not been

the case in the Soviet Union where according to Hospodka (1966) all the alcohol produced from beet molasses and sulphite liquor is produced by continuous fermentation. The multistage systems which are employed consist of up to 10 mechanically agitated vessels some of which are used for yeast propagation. A considerable volume of literature concerning continuous alcoholic fermentation has been published in the Soviet Union but it is difficult to obtain. Hospodka (1966) reviewed the Soviet literature available to that time but the more recent publications have been reviewed regularly (Malek and Ricica, 1966; 1968; 1969; 1970; Ricica, 1971; 1972; 1973).

Recently Goslich (1974) has claimed that it is possible to ferment unsterilized beet molasses continuously in a multivessel continuously agitated system. As a result of his investigations Goslich concluded that by employing at least six vessels it was possible to attain a rapid and efficient fermentation of unsterilized molasses.

A laboratory scale single stage continuously agitated apparatus for fermenting sterilised cane molasses was described by Bose and Ghose (1973) who demonstrated that the yields of alcohol from such a system were comparable to those obtained in batch fermentation.

The use of tubular fermenters for beer production has been investigated on the laboratory (Portno, 1967; Denshchikov, 1961a) and pilot and commercial scales (Klopper et alia, 1965; Ault et alia, 1969). In addition the A.P.V. tower fermenter has been employed to produce

vinegar charging wort (Greenshields and Smith, 1974) but there have been no attempts to apply this system to other alcoholic fermentation processes such as the production of ethanol from molasses. The production of ale and lager beer by continuous tower fermentation has been studied at the University of Aston in Birmingham (Greenshields and Smith, 1971; see Sections 1 and 3 in this thesis) and it was decided to extend the scope of the studies to include an investigation of the continuous fermentation of a beet molasses medium as might be used in a distillery. Experience gained during this study of continuous beer fermentation indicated that a number of yeasts in the Continuous Fermentation Culture Collection possessed the flocculence and fermentation characteristics required for the tower production of beer. Those yeasts which were known to be sedimentary under the conditions of beer brewing were inoculated into batches of a 27 g% w/v molasses based medium which contained approximately 15 g% w/v of fermentable sugar, and from the results of these fermentations one yeast was selected for further testing in the laboratory tower fermenter. Since there is no published information relating to the tower fermentation of beet molasses the objectives of this investigation were to establish i) whether it was possible to ferment beet molasses continuously by this process, ii) the concentration of medium which would allow a rapid and efficient fermentation.

Plate 2.

Laboratory Tower Fermenter employed for fermentation of molasses.

MATERIALS AND METHODS

i. The Molasses

The molasses was obtained during 1972 from the British Sugar Corporation refinery at Kidderminster. The results of basic analyses are given below:

Total solids	62.0 %
Total Nitrogen	1.47 g% (wet weight)
Total reducing substances (after hydrolysis)	54.96 g% (wet weight)

ii. Additions to Molasses

The medium prepared from the molasses was supplemented with:

Ammonium sulphate	B.D.H.
Sodium dihydrogen phosphate	B.D.H.

The amounts of each chemical employed in the media for various experiments have been detailed in the appropriate place.

iii. Fermentable sugar in molasses

The amount of rapidly fermentable sugar in the molasses based medium was determined by batch fermentations.

The medium had the following composition. All quantities are expressed as g/litre.

Beet molasses	91 g
$(NH_4)_2\ SO_4$	3.50 g
$NaH_2\ PO_4$	1.0 g

The pH was adjusted to 4.5 and the whole made up to 1 litre. Aliquots of 150 ml were pipetted into 250 ml conical flasks and autoclaved at 1.05 $kg.cm^2$ for 15 minutes.

Each flask was inoculated with 3 g of centrifuged yeast (CFCC 54) and incubated for 48 hours at 25^{o}C on a rotary shaker (Gallenkamp model IH 465) at 120 revolutions per minute.

The mean values of analysis quadruplicate fermentation are given below:

Initial reducing substances (after hydrolysis) 53.0 g/litre

Final reducing substances (after hydrolysis) 4.20 g/litre

$$\% \text{ fermentable sugar} = \frac{53.0 - 4.20}{53.0} \times 100$$

$$= 92.1 \%$$

iv. The yeast cultures

The master and working culture of the yeasts in the Continuous Fermentation Culture Collection were held on M.Y.G.P. medium (Wickerham, 1951) solidified with 1.8 % agar and stored at 4°C.

The details of individual yeast cultures are given below:

CFCC No.	Details
1	Saccharomyces cerevisiae NCYC 1026
3	Saccharomyces cerevisiae NCYC 1251
8	Saccharomyces cerevisiae NCYC 1119
10	Saccharomyces cerevisiae NCYC 1257
14	Saccharomyces cerevisiae NCYC 1269
15	Saccharomyces cerevisiae NCYC 1262
17	Saccharomyces cerevisiae NCYC 1258
18	isolates of Trumans brewery yeast
19	isolate of CFCC 10 - type 1 light
21	Saccharomyces cerevisiae NCYC 1251
25	Sacchayomyces cerevisiae NCYC 1068
27	24/8 E43 ex A.P.V. Company Limited
28	Saccharomyces cerevisiae NCYC 1270
31	isolate of CFCC 10 least flocculent) isolated
32	isolate of CFCC 10 intermediate flocculent) 27.12.67

CFCC No.	Details
33	isolate of CFCC 10 most flocculent) isolated 27.12.67
34	Saccharomyces cerevisiae NCYC 1260
36	Saccharomyces cerevisiae NCYC 1266
37	Saccharomyces cerevisiae A.J. 3075 ex A. Jorgenson
54	Saccharomyces diastaticus (G.606) ex A. Guinness and Sons Limited.

v. Analytical methods

The following methods were followed in the routine chemical analyses performed during this study.

a. Nitrogen

The nitrogen determinations were carried out by the standard micro-Kjeldahl method (Markham, 1942) using a catalyst containing selenium, copper sulphate and potassium sulphate catalyst in the ratios 1:5:32 parts by weight.

b. Reducing substances

Total reducing substances in the samples were measured by the Fehlings method (Lane and Eynon, 1923) or the ferricyanide method (Somogyi, 1945) after acid hydrolysis, which was achieved by adding 1 ml of concentrated hydrochloric acid for every 10 ml sample and heating in a water bath at 95^{o}C for 10 minutes.

c. Alcohol determination

The concentration of ethanol in the fermented medium was determined by the following distillation procedure:

A 100 ml sample of the medium was distilled slowly until approximately 60 ml of distillate had been collected in a 100 ml volumetric flask. The distillate was made up to 100 ml with distilled water and the specific gravity of

the distillate measured at 15°C with a Westphal type specific gravity balance (Stanton Instruments Limited). The concentration of ethanol in the distillate was determined by reference to 'Thorpe's Alcoholometric Tables' (Thorpe, 1915).

d. pH

pH was determined with a Pye model 98 pH meter.

The selection of yeasts by a batch fermentation technique

INTRODUCTION

The technique for selecting lager yeasts (Section 3) was based on assessment against two criteria.

i. flocculence

ii. batch fermentation characteristics

Similar tests have been employed to select a yeast for use in the laboratory tower fermenter.

i. The selection of flocculent (sedimentary) yeasts

The flocculence-sedimentation characteristics of the yeast were assessed by the method of Sharp (Greenshields et alia, 1972). Experience with ale and lager beer yeasts has shown that yeasts which obtain a modified Burns number less than 70 by this method are washed out of the tower fermenter at low volumetric efficiencies. Therefore only those cultures which obtained a modified Burns number greater than 70 were judged to be suitable for use in the tower fermenter. The results of the modified Burns test are recorded in Table 10.

ii. Batch fermentations with flocculent yeasts

The second stage of the selection procedure involved conducting batch fermentations with all the yeasts selected by the above method.

The composition of the medium employed in the fermentations is given below:

Beet molasses	271 g/litre
Ammonium sulphate	3.57 g/litre
Sodium dihydrogenphosphate	1.0 g/litre

The medium was adjusted to pH 4.5 with concentrated sulphuric acid and 150 ml aliquots dispensed into 250 ml conical flasks which

Table 10

The Modified Burn's Number of some yeasts in the Continuous Fermentation Culture Collection

a. Flocculent types

CFCC No.	Modified Burns No.	CFCC No.	Modified Burns No.
1	100	21	110
3	133	25	132
8	162	27	142
10	160	28	132
13	131	31	135
14	104	32	148
15	146	33	133
17	125	34	138
18	118	36	128
19	148	37	119
		54	170

b. 'Semi' flocculent types

41	52	75	46
42	43	76	57
48	39	82	49
		52	60

c. Non flocculent types

2	4	49	0
4	5	50	0
5	4	51	0
9	6	77	5
11	5	78	0
26	0.5	79	0

were then autoclaved at 1.05 kg/cm^2 for 15 minutes. The flasks were inoculated with 1.5 g of freshly grown centrifuged yeast and incubated at 25^{o}C for seven days. The yeasts were separated from the liquors by centrifugation (I.E.C. model B. 20) at 5000 rpm. for 3 minutes.

The clear liquors were analysed for total reducing substances and alcohol by the methods previously described (Page 128). The results are summarized in Table 11.

Three yeasts, CFCC 14, 15 and 54, produced liquors containing more than 6.50 g % w/v alcohol, but the alcohol yields (as defined below) were poor compared to those obtained with other yeasts which gave liquors containing less alcohol.

iii. Selection of a yeast for continuous fermentation studies

From the results in Table 11 it is apparent that the yeasts which produce the highest concentration of alcohol in the fermented liquor are not necessarily the most efficient when yield of alcohol is assessed against the amount of carbohydrate utilized. However, when selecting yeasts for use in an industrial alcohol process the efficiency of carbohydrate conversion may be secondary importance to the speed of fermentation and final concentration of alcohol attained.

When considering a yeast for use in the tower fermenter the flocculence-sedimentation characteristics of the yeast are of major importance.

Thus although yeast CFCC 14 gave the highest concentration of alcohol in the fermented liquor it was the least flocculent of the

Table 11

The sugar utilization and alcohol yield in liquor produced by batch fermentation with flocculent yeasts

Culture No.	Sugar utilization g % w/v	Alcohol content g % w/v	Alcohol yield g/100 g sugar fermented
1	11.90	5.70	48.9
3	12.18	5.70	46.9
8	12.52	5.76	46.1
10	12.16	5.76	47.2
14 *	14.47	6.74	45.6
15 *	14.53	6.54	45.0
17	12.12	5.76	47.5
18	11.96	5.51	46.1
19	12.23	6.02	49.1
21	10.75	5.13	48.1
25	10.96	5.13	46.8
27	12.89	5.76	44.7
28	13.59	6.34	46.8
31	12.11	5.70	47.1
32	11.81	5.57	46.4
33	12.98	6.02	46.4
34	12.81	6.02	47.0
36	12.81	6.02	47.0
37	12.50	6.02	47.2
54 *	14.48	6.54	43.37

Those cultures marked * were judged to be suitable for testing in the tower fermenter.

four yeasts under consideration. Similarly CFCC 28 was rejected because it gave a liquor with lowest alcohol concentration and was less flocculent than both CFCC 14 and 54. The yeast which was finally selected was CFCC 54 because it was considered that on balance the advantage of employing the more flocculent yeast would outweigh the minor difference in the yield of alcohol obtained. However, it was considered that yeast CFCC 14 would provide a satisfactory alternative to yeast CFCC 54.

The Continuous Fermentation of Beet Molasses

DEFINITION OF TERMS USED

Greenshields and Smith (1971) have reported that yeasts become physically or fermentation limited at a particular wort flow rate when fermenting brewer's wort. The flow rate at which the limitation occurs depends upon the yeast strain and the strength of wort (medium). Fermentation limitation is judged to have occurred when the amount of fermentable sugar remaining in the fermented medium rises above an acceptable level.

The molasses based medium employed in the following experiments conducted with the laboratory tower fermenter was of similar composition to that employed in the batch fermentations of the yeast screening experiment. It was established by experiment (see pages 126 and 127) that approximately 92% of reducing substances measured as invert after hydrolysis of the initial medium was fermentable sugar. It was calculated that 90% utilization of the total reducing substances corresponded to 97.8% utilization of fermentable sugar. Therefore it was decided that 'fermentation limitation' would be judged to have occurred when the yeast just failed to ferment 90 % of the total reducing substances in the medium.

The term 'volumetric efficiency' has been employed to describe the production rate of the laboratory tower fermenter. The calculation of volumetric efficiency has been described previously in Section 1. In this section of the thesis the term 'limiting volumetric efficiency' (LVE) has been used to denote the volumetric efficiency at which the fermenter could be operated and just maintain 90 % utilization of the total reducing substances in the medium, or that volumetric efficiency at which the yeast became physically limited.

The yield of alcohol has been expressed as 'g alcohol produced per 100 g of carbohydrate fermented'. The yield of alcohol predicted stoichiometrically is given by the equation

$$C_6H_{12}O_6 \longrightarrow 2\,C_2H_6O + 2\,CO_2$$

$$180\text{ g} \qquad 2 \times 46 + 2 \times 44$$

$\therefore$ yield per 100 g CHO fermented $= \frac{92}{180} \times 100 = 51.1$ g

However, this calculation disregards the fact that some carbohydrate is utilized in yeast growth. In distillery practice the yield of alcohol normally attained is 47.1 g per 100 g of fermented monosaccharide (Harrison and Graham, 1970) which is 92 % of the theoretical yield. The remaining carbohydrate is incorporated as yeast material or directed into alternative metabolic pathways to yield glycerol and succinic acid.

Total reducing substances (TRS) - the reducing substances (measured as invert sugar after hydrolysis) present in the medium before fermentation.

Residual reducing substances (RRS) - the reducing substances (measured as invert sugar after hydrolysis) present in the fermented medium.

Fermented sugar - calculated as the difference between T.R.S. and R.R.S.

Fermentable sugar (FS) - has been calculated as 92.1 % of T.R.S. for experiments i) and ii) and 94.7 % for experiment iii).

Experiment 1.

The fermentation of Molasses based Medium

MATERIALS AND METHODS

A medium of the following composition was fermented continuously in the tower fermenter:

Molasses (beet)	182 g/litre
Ammonium sulphate	3.57 g/litre
Sodium dihydrogen phosphate	1.0 g/litre

The ammonium sulphate was added to adjust the C:N ratio to 11.5:1 to ensure that the yeast was not nitrogen limited and that sufficient assimilable nitrogen was available for growth. The pH of the medium was adjusted to 4.5 before autoclaving in 20 litre batches at 1.05 kg/cm^2 for 30 minutes. This media contained 100 g reducing substances per litre.

RESULTS

The results of analysis upon individual samples have been tabulated in the Appendix, Tables A 9 and A 10.

a. Utilization of fermentable sugar (F.S.)

The utilization of fermentable sugar has been reported as a percentage of the fermentable sugar and also as a percentage of the total reducing substances (T.R.S.). This information is presented in Table 12 below.

Table 12.

Sugar utilization by CFCC 54 during continuous fermentation at 20°C

Volumetric Efficiency	% Utilization of total reducing substances	% Utilization of fermentable sugar
0.9	91.8	99.6
1.46	91.3	98.8
2.9	91.5	99.2
3.5	91.36	99.1
5.4	89.2	96.8

The above results indicated that the tower fermenter could be operated at volumetric efficiencies up to 3.5 and maintain 99 % utilization of the fermentable sugar in the medium. At volumetric efficiency 5.4 the utilization of the fermentable sugar fell to less than 97 %.

The limiting volumetric efficiency has been defined as that volumetric efficiency at which 97.8 % utilization of fermentable sugar was just maintained. Inspection of the above results indicated that the limiting volumetric efficiency lay between volumetric efficiencies of 3.5 and 5.4. This value could be estimated by interpolation, and was calculated to be 4.67.

b. Alcohol yield

The yield of alcohol has been expressed as g of alcohol produced per 100 g of sugar fermented. The mean values for the yields of alcohol at various volumtetric efficiencies are presented below.

Table 13.

Alcohol yield at various volumetric efficiencies

Volumetric Efficiency	Alcohol yield g/100 g sugar fermented	Range of Values
0.9	44.1	43.8 - 51.1
1.46	44.6	42.5 - 49.9
2.9	39.6	37.9 - 40.8
3.5	41.1	37.5 - 44.0
5.4	40.5	37.4 - 42.3

Inspection of the results of analysis upon the individual samples indicated a wide range of values for alcohol yield, and therefore any conclusion drawn from these results had to be regarded with caution. However, a trend towards reduced alcohol yield at the higher volumetric efficiencies was evident. A second experiment was conducted and the results are presented below.

Table 14.

Alcohol yield at various volumetric efficiencies

Volumetric Efficiency	Alcohol yield g/100 g sugar fermented	Range of Values
1.20	40.1	37.4 - 43.5
1.55	42.2	41.6 - 43.0
2.5	41.5	41.0 - 42.0
3.2	43.5	42.7 - 44.0

These results represent if anything a reverse trend to that observed in the first experiment. However the range of values obtained at any particular volumetric efficiency was smaller.

In view of the contradictory nature of these results it seems likely that the alcohol yield was independent of the volumetric efficiency at which the fermenter was operated. Some of the variation in values for alcohol yield may be attributed to the method of estimating alcohol. It should be possible to obtain more accurate estimates of the alcohol concentration and yield by techniques such as gas liquid chromatography or by microdiffusion analysis. The studies of Bose and Ghose (1973) upon the performance of a continuous single stage homogeneous fermentation of cane molasses indicated a trend towards improved yield at higher dilution rates though there were insufficient data to allow any firm conclusions to be drawn.

c. pH

The pH of the inflowing medium was maintained between 4.5 and 4.7 and the pH of the fermented liquor was usually approximately 0.15 of a pH unit lower. The range of pH observed in the fermented liquor was between pH 4.32 and pH 4.7 but the majority of samples had a pH between 4.45 and 4.5.

It was evident from these results that the medium was well buffered. Harrison and Graham (1970) state that it is advantageous to maintain the pH below 4.7 because at higher pH values the yield of alcohol falls as more glycerol and organic acids are produced.

Experiment ii.

The fermentation of media of various concentrations

The following experiment was conducted to investigate the effects of fermenting media at various concentrations and to determine the limiting volumetric efficiency with each medium.

a. The media

The media was prepared in 20 litre batches. The concentration of the medium was altered by adjusting the amount of the molasses component. The weights of molasses employed in each medium have been given below.

Weight of molasses g/litre	Total reducing substances g/litre
182	100
274	150
318	175
364	200

The other components in the media were present in the following amounts:

Ammonium sulphate 3.57 g/litre

Sodium dihydrogen phosphate 1.0 g/litre

The pH was adjusted to 4.5 by addition of sulphuric acid and the media autoclaved at 1.05 kg/cm^2 for 30 minutes.

RESULTS

The results of the analysis of individual samples have been recorded in the Appendix (Table A 11).

a. The limiting volumetric efficiency

The limiting volumetric efficiency was established when fermenting each medium. The results have been tabulated below:

Table 15.

The limiting volumetric efficiencies and medium concentration

Medium concentration molasses g/litre	Limiting volumetric efficiency
182	4.67
274	2.5
318	2.0
364	1.1

The limiting volumetric efficiency was dependent upon the concentration of the medium and fell almost in a direct proportion to the increase in concentration of the medium.

b. Sugar utilization rate at the limiting volumetric efficiency

The sugar utilization rates at the limiting volumetric efficiencies with the media of various concentrations were calculated and have been presented overleaf.

Table 16.

Sugar utilization rate at the limiting volumetric efficiency

Medium concentration molasses g/litre	Sugar utilization rate g/hr
182	87.5
274	68.8
318	62.5
364	40.2 *

* At volumetric efficiency 1.1

When fermenting media containing 318 g molasses/litre the rate of sugar utilization was 28.6 % lower than that observed when fermenting the most dilute medium (182 g molasses/litre).

When the concentration of the medium was increased to 364 g molasses/litre the rate of sugar utilization fell to 45.9 % of that observed when fermenting the least concentrated medium.

c. Alcohol yield

The yield of alcohol from the various media have been tabulated below.

Table 17.

Medium concentration molasses g/litre	Mean alcohol yield g/100 g sugar fermented	Range
184 *	41.2	37.5 - 44.0
274	42.8	39.9 - 45.3
318	45.0	43.8 - 45.9
364	44.2	42.1 - 46.4

* At volumetric efficiency 3.5

The maximum yield of alcohol was obtained when fermenting a medium containing 318 g molasses/litre.

The overall rates of alcohol production at the limiting volumetric efficiencies were calculated from the sugar utilization rates and alcohol yields.

Table 18.

Medium concentration molasses g/litre	Rate of alcohol production g/hr
* 182	27.6
+ 182	40.7
274	29.5
318	28.5
364	17.8

* At volumetric efficiency 3.5

\+ At volumetric efficiency 5.4

The rate of alcohol production at the limiting volumetric efficiency when fermenting the medium containing 182 g molasses/ litre was calculated by making the following assumptions.

a. The limiting volumetric efficiency was 4.67

b. The utilization of reducing substances was 90 g/litre at the limiting volumetric efficiency.

c. The alcohol yield was 40.0 g/100 g reducing substances utilized.

$\therefore$ Medium flowrate at volumetric efficiency 4.67 = 0.972 litre/hr

$\therefore$ Sugar utilized/hour = 0.972 x 90.0

= 87.6 g/hr

$\therefore$ Alcohol production rate = 87.6 x 0.40 g/hr

= 35.04 g/hr

The above figure was probably an underestimate of the alcohol production rate since the figure for alcohol yield at the limiting volumetric efficiency was pessimistic when compared with the mean value for alcohol yields at volumetric efficiencies 3.5 and 5.4. It seems reasonable, therefore, to assume that the overall rate of alcohol production was highest when fermenting the most dilute medium.

The above results and calculations indicate that the fermentation of more concentrated media gave an improved alcohol yield, but only at the sacrifice of the overall rate of alcohol production. The mode of operation of a commercial fermentation system will depend upon economic considerations. The advantages of rapid fermentation when using a dilute substrate will have to be weighed against the cost of distillation and removing proportionally larger quantities of water. This experiment has illustrated that the tower system can be employed to ferment substrates of widely different concentrations but that the overall rate of product formation depends upon the concentration of the substrate.

d. pH

The pH of the inflowing medium was maintained between 4.5 and 4.7. The pH of the fermented liquor depended upon the concentration of the medium. This has been summarized below.

Table 19.

Medium concentration molasses g/litre	Medium pH	Liquor pH
182	4.5 - 4.72	4.32 - 4.7
274	4.62	4.48 - 4.52
318	4.5	4.6 - 4.73
364	4.55	4.70 - 4.72

The pH of the fermented liquor originating from the two more dilute media was in general lower than that of the media, but the trend was reversed in the liquor arising from the concentrated media.

e. Nitrogen utilization

The total amounts of nitrogen in the medium and the fermented liquor were measured routinely. No pattern of nitrogen utilization was apparent and when the nitrogen uptake from the medium was calculated as a percentage of the nitrogen supplied a wide range of values was obtained.

Individual values for the nitrogen content of the medium and liquor have been presented in the Appendix (Table A 12).

Whilst fermenting the medium containing 182 g. molasses a net loss of nitrogen from the medium was usually observed but the values varied between 0 and 13.2 % of the nitrogen supplied. On five occasions the nitrogen content of the liquor was greater than that of the medium. It was not possible to attribute this to any particular event such as a change in the medium flow rate. This type of event was not observed before day 44 of the continuous operation but occurred frequently thereafter, particularly when fermenting the more concentrated media. By day 44 the yeast concentration in the tower was very high and did not appear to increase thereafter. It seems probable therefore that the yeast concentration reached the limit which could be maintained in the fermenter under the prevailing conditions and that a balance between yeast growth and death was established.

When the concentration of the medium was increased the balance was upset and the death rate of the yeast increased and caused a net loss of nitrogen from the yeast mass to the medium. It was not possible to calculate a nitrogen balance for the reactions within the tower fermenter because it was impossible to establish the

yeast mass with accuracy.

It was clear that nitrogen supplementation of the medium was unneccessary since the net uptake of nitrogen even during the early stages of the experiment was rarely greater than 10 % of the total nitrogen supplied, and never exceeded the value for nitrogen added to the medium in the form of ammonium sulphate.

Experiment iii

The fermentation of sucrose supplemented media

INTRODUCTION

In the previous experiment it was established that a considerable reduction in the rate of sugar utilization and alcohol production occured when the concentration of the medium was increased. Therefore the following experiment was conducted to investigate whether the sugar utilization rate and alcohol yield could be improved by modification of the medium. It was decided to increase the proportion of fermentable substances in the medium by the addition of sucrose.

MATERIALS AND METHODS

i. The Medium

The composition of the medium was based upon that of the most dilute medium used in the previous experiment.

Sucrose is not utilized directly by yeast but is hydrolysed extracellularly to its component monosaccharides. During hydrolysis there is an apparent 'gain' in the weight of sugar and the amount of this gain can be calculated.

$$C_{12}H_{22}O_{11} \xrightarrow[\text{(hydrolysis)}]{+ H_2O} 2\,C_6H_{12}O_6$$

$$342 + 18 = 360$$

$\therefore$ 342 g sucrose yield 360 g of reducing monosaccharides.

$\therefore$ 50 g of monosaccharide result from the hydrolysis of

$$50 \times \frac{342}{360}\ \text{g sucrose}$$

$$= 47.51\ \text{g}.$$

The medium prepared to the formula given below contained approximately 150 g reducing substances/litre.

Beet molasses	182 g/litre
Sucrose	47.5 g/litre
Ammonium sulphate	3.57 g/litre
Sodium dihydrogen phosphate	1 g/litre
C : N ratio	20 : 1

The pH was adjusted to 4.5 with sulphuric acid prior to autoclaving at 1.05 kg/cm^2 for 30 minutes.

Fermentable substances in the medium

A batch of medium was made to the formula above and diluted with distilled water to give a concentration of total reducing substances of 50.8 g/litre. Aliquots of 150 ml were pipetted into 250 ml conical flasks and autoclaved at 1.05 kg/cm^2 for 15 minutes.

Each flask was inoculated with 3 g of centrifuged yeast (CFCC 54) and incubated for 48 hours at 25°C on a rotary shaker (Gallenkamp model I.H. 465) at 120 revolutions per minute. The mean values of analysis upon quadruplicate fermentation are given below.

Initial reducing substances		50.8 g/litre
Residual reducing substances after fermentation		2.7 g/litre
% of fermentable reducing substances	=	$\frac{2.7}{50.8} \times 100$
	=	94.69

RESULTS

The results of analysis of individual samples have been tabulated in the Appendix (Table A 13).

a. Limiting volumetric efficiency

The mean values for the utilization of reducing substances in the fermented liquor produced at various volumetric efficiencies have been produced on Graph 17.

In the previous experiments with molasses media the limiting volumetric efficiency was determined as that volumetric efficiency at which 90% utilization of the reducing substances was just maintained. This corresponded to 97.8 % utilization of fermentable sugar, and therefore the same minimum value for fermentable sugar utilization was applied in this experiment.

The mean values for substrate utilization at specific volumetric efficiencies are tabulated below:

Table 20

Sugar utilization at various volumetric efficiencies

Volumetric Efficiency	% Utilization of fermentable sugar
1.2	99.6
2.25	98.1
2.8	97.9
7.2	91.1

The results for individual samples were analyses by the method of least squares to give the line of best fit. The equation of the line and the coefficient of correlation have been given below:

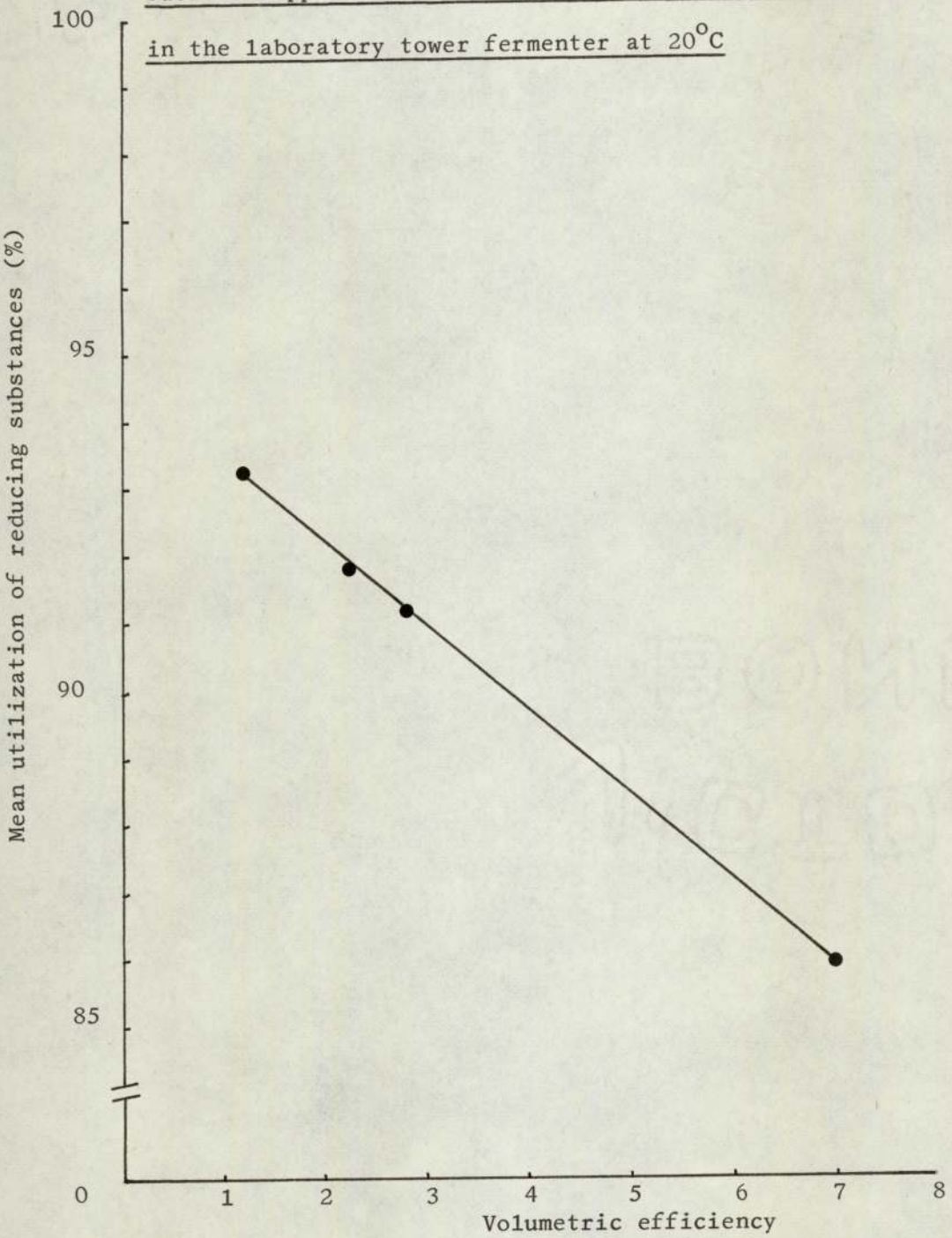
$$y = -1.44x + 101.56$$

$$r = 0.98$$

The limiting volumetric efficiency was calculated as 2.61 by

Graph 17.

The relationship between utilization of reducing substances and volumetric efficiency when fermenting sucrose supplemented molasses medium with yeast CFCC 54 in the laboratory tower fermenter at 20°C



substitution of the value 97.8 for y in the equation.

The limiting volumetric efficiency when fermenting the sucrose supplemented media was not appreciably different from the limiting volumetric efficiency (2.5) for the fermentation of an unsupplemented medium of similar concentration. Therefore it was not apparent that the limiting volumetric efficiency was mainly dependent upon the amount of the fermentable substance in the medium, and not to the proportion of the non-fermentable molasses component.

b. Sugar utilization rate

The sugar utilization rate at the limiting volumetric efficiency was calculated making the following assumptions:

i. The limiting volumetric efficiency was 2.61.

ii. The yeast utilized 98.7 % of the fermentable substances at the limiting volumetric efficiency.

iii. The medium contained 150 g total reducing substances/litre.

∴ Flow rate at the limiting volumetric efficiency was

$$\frac{2.61 \times 5}{24} \text{ litres/hour}$$

$$= 0.544 \text{ litres/hour}$$

iv. The proportion of fermentable sugar was 94.6 % of the total reducing substances.

∴ Utilization of fermentable substances at the L.V.E. $= 150 \times \frac{94.6}{100} \times \frac{97.8}{100}$ g/litre

$$= 138.7 \text{ g/litre}$$

∴ Sugar utilization rate $= 138.7 \times 0.544$

$$= 75.3 \text{ g/hour}$$

This rate of fermentation was greater than that obtained when fermenting an unsupplemented molasses medium of similar concentration, but less than that obtained when fermenting a molasses medium containing 182 g molasses/litre.

At volumetric efficiency 7.2 the utilization of fermentable sugar fell to 91 %, but the overall rate of utilization of fermentable sugar rose to 192.5 g/hour, more than double that at the limiting volumetric efficiency, and corresponded to a specific utilization rate of 0.497 g fermentable sugar/g dry yeast/ hour. Attempts to increase the volumetric efficiency above 7.2 resulted in excessive washout of the yeast.

c. Alcohol yield

The alcohol yields at various volumetric efficiencies are presented below.

Table 21.

Alcohol yield at various volumetric efficiencies

Volumetric Efficiency	Alcohol yield g/100 g sugar fermented
1.2	42.1
2.25	48.3
2.8	46.3
7.2	45.5

The results indicated that the alcohol yield was minimal when the fermenter was operated at a low volumetric efficiency. Above volumetric efficiency 2.0 the alcohol yield was greater than 45.0 g/100 fermented reducing matter. This yield represented an improvement of between 8.1 and 12.8 % upon the yield obtained when fermenting an unsupplemented molasses medium at the limiting volumetric efficiency.

d. pH

The pH of the medium flowing into the base of the tower fermenter was between 4.6 and 4.65. During fermentation the pH

dropped slightly and was between 4.4 and 4.51 in the liquor leaving the head of the fermenter. The drop in pH was of the same magnitude as observed in Experiment 1 when fermenting the molasses medium containing 182 g molasses/litre. Thus the change in pH is dependent upon the concentration of molasses employed and reflects the buffering capacity of the medium.

e. Temperature

The rate of fermentation by yeast increases with increasing temperature to an optimum which normally occurs at between 30°C and 40°C.

The previous experiments were conducted at 20°C and it was decided to investigate whether increasing the temperature would have a marked effect upon the rate of fermentation. However, raising the temperature causes an increase in the rate of evaporation and to avoid excessive loss of alcohol it has become standard practice within the fermentation industry to maintain the temperature below 30°C.

Therefore it was decided to operate the fermenter at a temperature of 28°C and volumetric efficiency 7.2.

The results of analyses upon individual samples have been recorded in the Appendix but have also been presented below in a summarized form.

Table 22

Sugar utilization and alcohol yield at 20°C and 28°C

Temperature °C	% Utilization fermentable sugar	Alcohol yield g/100 g fermented sugar
20	91.1	45.5
28	95.6	44.7

At the higher temperature the sugar utilization was greater

and the rate of sugar utilization rose from 192.5 g/hour to 204.2 g/hour an improvement of 6.02 %. Conversely the yield of alcohol was reduced by 0.8 % at the higher temperature.

Nevertheless the advantage gained from improved substrate utilization more than outweighed the loss in alcohol yield so that the alcohol production rate was increased 4.2 % at the higher temperature.

Morphology of CFCC 54 in the tower fermenter

The morphological form which a yeast assumes in the tower fermenter is the major factor governing the performance of the system. The yeast chosen for the investigation into continuous fermentation of molasses was selected because it was the most flocculent of the yeasts which produced a liquor containing greater than 6.5 % w/v alcohol. The behaviour of the yeast was observed during the course of the experiments performed with the laboratory tower fermenter operating at various volumetric efficiencies with media of differing concentrations and composition.

a. Morphology when fermenting molasses media

The morphology of CFCC 54 was dependant upon the volumetric efficiency at which the tower fermenter was operated. The sequence of events described below was observed when fermenting a medium containing 182 g molasses/litre.

When operating the tower fermenter at volumetric efficiencies below 1.5 the yeast in the 2.5 cm section was closely packed to form a solid bed, which contained gas bubbles of various sizes (Plate 3). A dense plug of yeast formed at the base of the fermenter just above the medium inlet point and channelling of the medium and fermentation gas was observed. This plug, 10 - 13 cm deep, was stable at all volumetric efficiencies up to 3.6 when the yeast began to separate into discrete particles of 0.3 - 0.4 cm diameter which exhibited some random motion. The particles were observed to remain at the base of the fermenter at all the rates of operation investigated (up to volumetric efficiency 5.4).

At volumetric efficiency 2.9 the yeast in the main part of the 2.54 cm section began to disperse to form aggregates of 0.2 - 0.3 cm diameter (Plate 4). Above volumetric efficiency 4.0 the yeast

Plate 3.

The gross morphology of yeast CFCC 54 in the 2.54 cm diameter section of the laboratory tower fermenter when fermenting medium containing 182 g molasses/litre at 20°C and volumetric efficiency 1.5

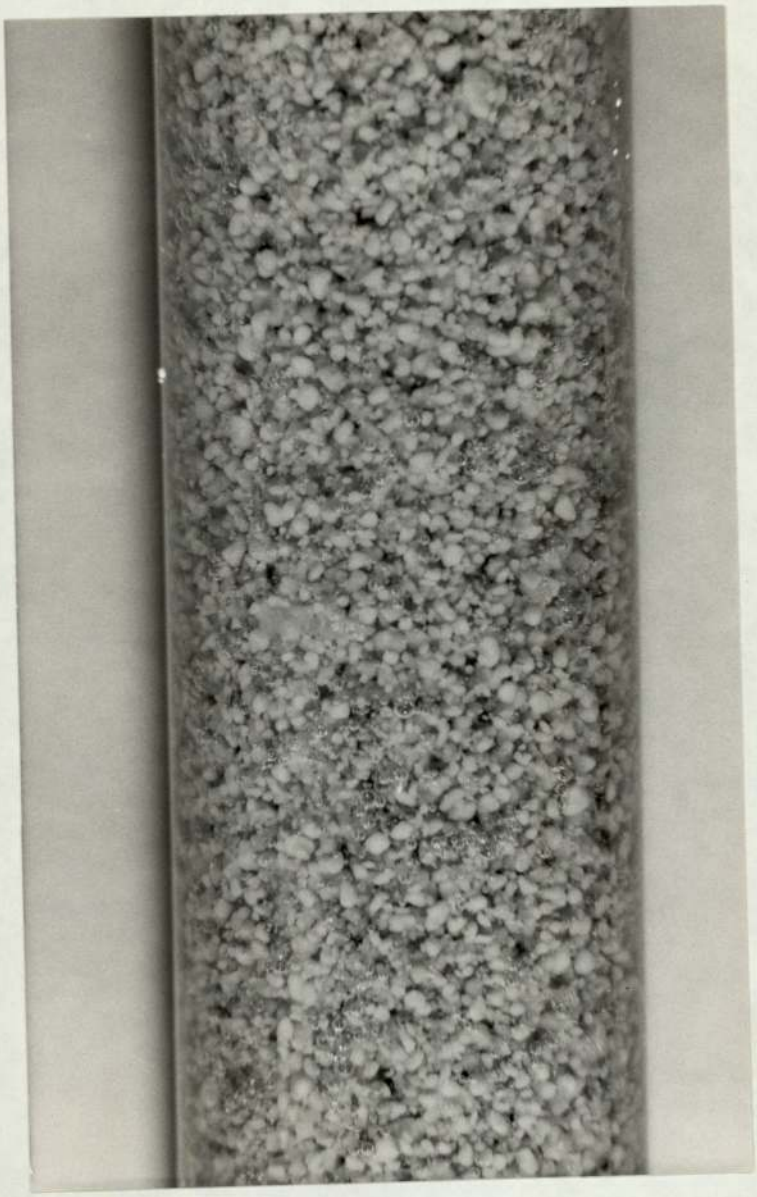


Plate 4.

The gross morphology of yeast CFCC 54 in the 2.54 cm diameter section of the laboratory tower fermenter when fermenting medium containing 182 g molasses/litre at 20°C and volumetric efficiency 2.9

mass in this section was unstable and the sequence of events illustrated in Plates 5 - 8 was observed.

The yeast in the dispersed state (Plate 5) began to aggregate into larger particles which hindered the escape of fermentation gas (Plates 6 - 8). Eventually solid plugs of yeast formed and these were forced up the expanded section of the fermenter upon the cushions of gas. Upon reaching the head of the fermenter the violent agitation caused by the release of the gas detached lumps of the yeast up to approximately 1 cm diameter which then fell back into the 2.54 cm section and de-flocculated. Eventually the 2.54 cm section was filled with dispersed yeast (Plate 5). The complete cycle of events took approximately 5 minutes.

The yeast in the expanded section of the fermenter remained in a single densely packed lump but expanded to a 'spongy' texture at the highest rate of operation (volumetric efficiency 5.4).

The morphology observed when fermenting a more concentrated medium (274 g molasses/litre) was similar to that which has been described previously except the change from the 'packed bed' situation to one in which the yeast formed discrete particles occurred at around volumetric efficiency 2.5. When volumetric efficiency 2.8 was reached the yeast mass in the head section began to break up into lumps up to 0.5 cm diameter. When the medium concentration was raised to 364 g molasses/litre the maximum operating rate of the fermenter was reduced to volumetric efficiency 1.2. The morphology of the yeast was similar to that observed when operating at volumetric efficiency 2.8 with medium containing 274 g molasses/litre.

b. Morphology when fermenting molasses-sucrose medium

The morphology of the yeast when fermenting sucrose supplemented

The sequence of events observed when the tower fermenter was operated at volumetric efficiencies 4.0 with yeast CFCC 54 fermenting molasses medium at 20°C.

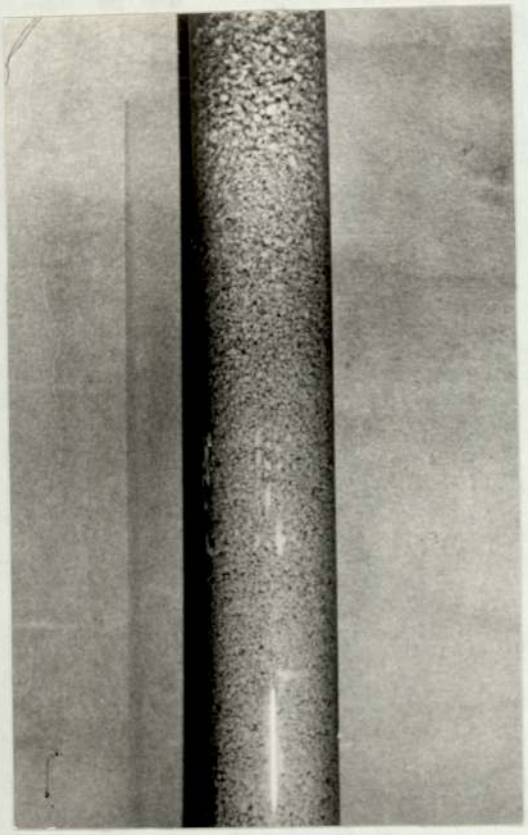


Plate 5

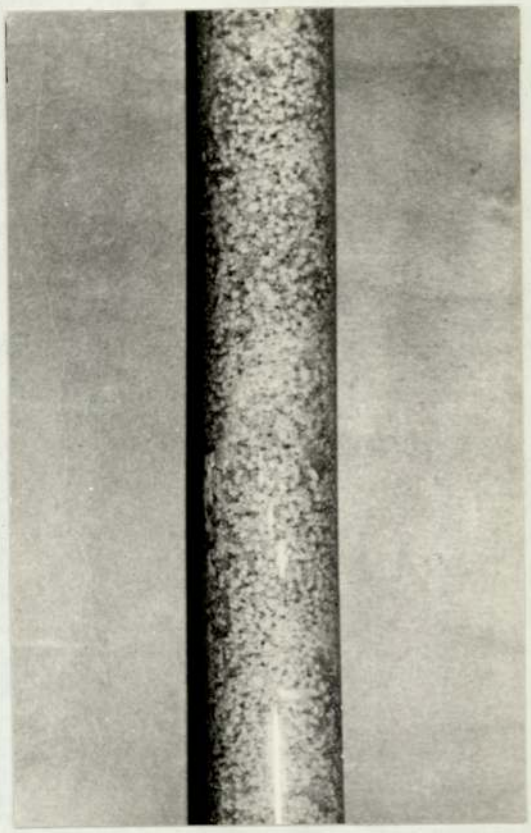


Plate 6

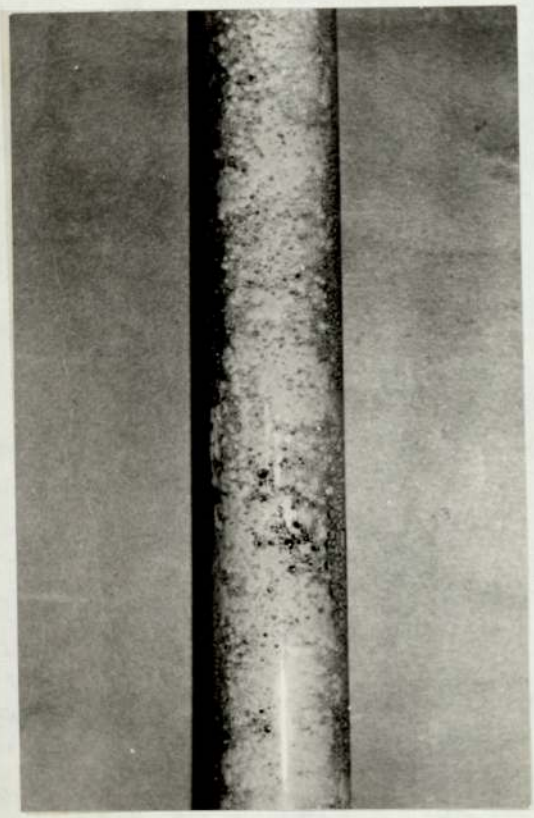


Plate 7

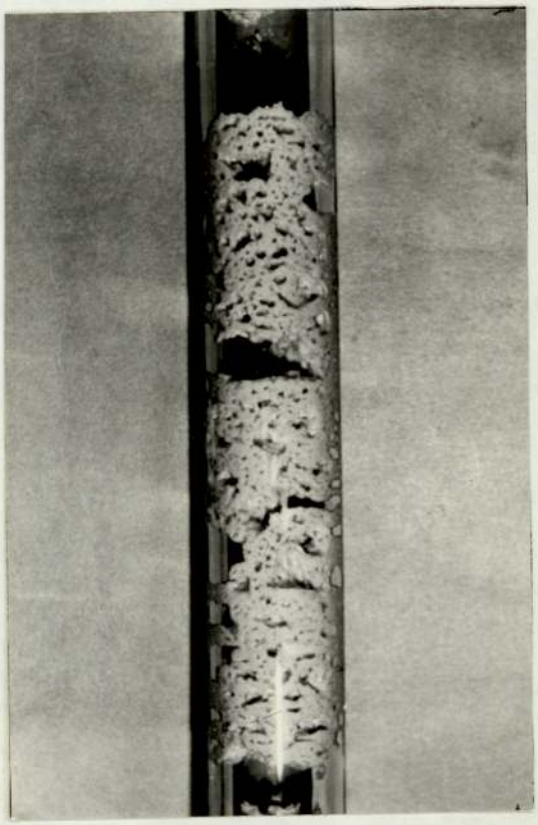


Plate 8

medium containing 274 g molasses/litre was similar to that observed when fermenting molasses medium of a similar concentration.

During the course of the experiment the tower fermenter was operated at a volumetric efficiency of 7.2. At this rate of operation the yeast mass in the head of the fermenter had a spongy appearance and exhibited a rolling motion. Ocassionally lumps of yeast would break off from the main mass and float to the surface of the liquid.

When the operating temperature of the fermenter was raised from 20^{o} to $28^{o}C$ the yeast mass in the head of the fermenter lost cohesion and lumps up to 2 cm diameter floated into the yeast seperator causing intermittent blockage of the outlet pipe. At the higher temperature a substantial amount (approximately 25 %) of yeast was washed out of the fermenter. These observations indicate that the behaviour of the yeast follows a general pattern. However, the morphological form which prevails is dependent upon the interaction of three factors.

i. The medium flow rate

ii. The medium concentration

iii. The operating temperature

DISCUSSION AND CONCLUSIONS

The results of this experiment showed that a rapid rate of production could be attained in a tower fermenter with liquid residence times as low as 2.6 hours and better than 90 % utilization of fermentable carbohydrate. The rate at which the fermenter could be operated was governed by the requirement for almost complete utilization of the fermentable carbohydrate and not by physical considerations such as washout of the yeast.

The other main point of interest was that the alcohol yield when fermenting the sucrose supplemented medium was better than that achieved when fermenting an unsupplemented molasses medium of a similar concentration. The yield of alcohol normally attained in commercial batch fermentation is 47.1 g alcohol per 100 g of sugar fermented, (Harrison and Graham,1970) but the yield obtained by the tower fermentation process was consistently lower than this (39.1 - 43.0 g per 100 g sugar fermented). The reasons for the inferior yield have not been established but from the results obtained in this experiment it is apparent that some improvement in yield may be expected by modifying the medium. A further improvement in yield may be expected by optimization of the aeration rate. Oura (1973) investigated the effect of aeration upon the growth of a strain of Saccharomyces cerevisiae and confirmed that ethanol production was greatest under conditions of total anaerobiosis. During the early stages of this work (Experiment 1) it was established that aeration of the fermenter at low rates (120 ml/min) had a beneficial effect upon sugar utilization. Attempts to establish the uptake of oxygen by the yeast within the tower fermenter were unsuccessful because of vast fluctuations in the rate of gas evolution due to the 'pocketing' of the gas within the fermenter, and so the effect of limited

aeration in tower fermenters remains unclear. However, the results of the experiment indicated that total anaerobiosis led to inefficient uptake of sugar and hence limited the production rate of the fermenter.

It has been pointed out by Shore and Royston (1968) that the tower fermenter is expensive compared to similar batch fermenters and in order to compete, the system has to operate at a high volumetric efficiency. A loss in alcohol yield may be unaviodable therefore, if the fermenter is to be operated at high volumetric efficiencies. The results reported by Yarovenko and Nakmanovich (1973) for the pilot scale multistage continuous fermentation of a wheat mash indicate a yield of 48.5 g alcohol per 100 g of hexose sugar fermented. Yields up to 47 g alcohol from 100 g of sugar were obtained by Bose and Ghose (1973) in a laboratory scale single stage apparatus of the chemostat type. It seems probable that the yields of alcohol from the tower fermentation process may be improved by further investigation of the effects of medium composition and aeration but at the present stage of development the process must be considered inefficient compared to the simpler batch and more complex multistage stirred system which are in use at the present. It requires a careful economic analysis to determine whether the benefits of a comparatively simple single stage tower system would compensate for the limitations which have been discovered in these experiments.

GENERAL DISCUSSION OF ALL SECTIONS

Until the beginning of this century the production of potable alcohol in the form of beers, wines and spirits was, and to a limited extent may even now be regarded as an 'art' rather than a science. It was the discoveries of Pasteur (1876) which opened the way to scientific investigation of alcoholic fermentation and since that time there have been innumerable investigations of every aspect of the process, and progress has been rapid. Indeed within 25 years of Pasteur's discoveries continuous methods for the production of beer had been suggested (Delbruck, 1892).

To operate a continuous process successfully requires a basic knowledge of the microbiological and biochemical processes involved. In addition, it is necessary to prevent the entry of unwanted micro-organisms, and in this respect equipment, such as open plate coolers and open fermentation vessels are inadequate. Similarly the media to be fermented requires more rigorous treatment to ensure that it is sterile.

It was the lack of fundamental knowledge which led to the failure and subsequent rejection of continuous fermentation processes by the brewing industry.

The introduction of more complex fermentation processes such as the production of antibiotics led to collaboration between microbiologists and biochemists and chemical engineers (Malek, 1966). The result was a new science, bioengineering.

The knowledge which was gained has been adopted and employed throughout the fermentation industries, although progress has been slower in the brewing industry.

In 1950 the theory of continuous culture was established by Monod and by Novick and Szilard, and later developed by Herbert et

alia, (1956). These advances reawakened interest in continuous processes for the brewing industry. Most of the modern continuous alcoholic fermentation systems are multistage homogenous systems employing mechanically agitated vessels (Herbert, 1961 - see Introduction Page 8). However, in one instance a single stage tubular reactor has been employed for the production of ale beer in Britain. The tubular reactor has long been employed by the chemical industry (Denbigh, 1966) and perhaps it was inevitable that a tubular form of fermenter should be proposed for the fermentation of beer.

The tubular reactor in brewing behaves as a heterogeneous system, with a progression from wort to fully fermented beer within the single vessel. In theory at each point in the fermenter there exists a steady state corresponding to a certain stage in the batch fermentation process. Tubular reactors exhibiting fermentation gradients were operated successfully in the laboratory (Denshchikov, 1961a;Portno, 1967) but the system proved unsuitable for commercial operation (Portno, 1974).

A vertical tubular reactor for the continuous fermentation of brewers' wort, the Tower Fermenter, was patented by Shore and Watson (1963) for the A.P.V. Company Limited. Early experiments with the tower fermenter for the production of ale and lager beers were described by Klopper et alia, (1965). The production of ale beer in a commercial scale fermenter was described by Ault et alia, (1969), and a number of A.P.V. Tower Fermenters are installed in a new brewery for Bass Charrington Limited. Although Tower Fermenters have proved successful in ale brewing there have been no reports of the commercial production of lager beer by this process, mainly because of difficulties in obtaining the desired flavour.

An aerated form of the tower fermenter has been employed for the production of biomass by batch (Morris, 1972) and continuous fermentations (Pannell, 1974). Recently a pilot scale tower fermenter has been employed as a continuous acetator for the production of vinegar (Greenshields and Smith, 1974). However, it is in the field of anaerobic fermentation (i.e. beer production) that the tower fermenter has been most widely and successfully employed to date. The kinetics of the tower fermentation processes are largely unknown and in consequence the performance of a continuous tower fermenter has to be established by experiment. Indeed it may prove impossible to establish the performance of a tower fermenter by any means other than experiment since the morphological behaviour of the microorganisms is difficult to predict accurately.

The introduction of the continuous fermentation process has thrown up a number of problems which are closely interrelated but may be conveniently considered to be one of two types:

i. Microbiological

ii. Engineering

One of the microbiological problems in beer brewing has been the selection of a suitable yeast. (To be economically competitive the tower fermenter has to operate at a high volumetric efficiency. The required volumetric efficiency can only be attained when a high concentration of yeast has been established within the fermenter). Early experiments (Klopper, et alia, 1965) indicated that the necessary concentration of yeast could only be established by employing a flocculent yeast which was selected by experiment in pilot scale equipment. Experiments with a laboratory tower of 1.83 m indicated that homogeneous conditions developed. This

problem was overcome by extending the fermenter to a height of 4.57 m. However, a unit of this size is too large to operate in a laboratory. In order to undertake a yeast selection programme in the laboratory the fermenter has to be of a size which can be conveniently operated with laboratory facilities. The fermenters described in this work were designed specifically for operation in a laboratory to facilitate a yeast selection programme.

The theory of tower fermenters for beer production assumes that the mode of operation is similar to that in plug flow system except that the three phases, solid, liquid and gas have different residence times. In practice it is evident that the assumption of plug flow characteristics is an over simplification. The experiments reported in this and previous work (Greenshields and Smith, 1971) have indicated that the yeast mass does not behave as if it were a fixed catalyst bed but that constant and often quite substantial movement of the yeast occurs. The factors influencing the mixing and fluidization - sedimentation phenomena in a tower fermenter have been discussed by Smith and Greenshields (1973), who nevertheless, consider that the flow is not far removed from plug flow.

The work presented in Section I indicated the procedure which was developed to select yeasts by use of a laboratory scale tower fermenter.

It has been shown that the fermentation gradient within the laboratory tower fermenter is similar to that observed in pilot (Klopper et alia, 1965) and full scale tower fermenters (Ault et alia, 1969).

The major problem associated with continuous tower fermentation of lager beer has been the high levels of vicinal diketones found in the finished beer. It has been possible to operate the laboratory

tower fermenter over a wide range of volumetric efficiencies and with wort of various specific gravities.

The experimental data indicate that the major influences upon the levels of vicinal diketones are the temperature of fermentation and the volumetric efficiency at which the fermenter is operated; low volumetric efficiencies and high temperatures give beers containing low levels of vicinal diketones. When high gravity worts are fermented the tower can only be operated at a low volumetric efficiency and hence the level of vicinal diketones in the beer is reduced to an acceptable level.

It has been shown that the presence of gaseous oxygen cannot be permitted if the level of vicinal diketones is to be controlled. Although it was possible to determine trends by statistical analysis, large fluctuations in the level of vicinal diketones were observed under certain conditions. It is possible that the fluctuations were a result of longitudinal mixing which occured because of the truncated form of, and absence of baffles in, the laboratory tower fermenter.

It has been reported that a diacetyl gradient exists within a pilot scale fermenter (Klopper et alia, 1965) and that the level of vicinal diketones in the beer was relatively constant over a wide range of wort flow rates.

It is suggested that a temperature gradient with perhaps a difference of 10°C (say 15°C at the bottom and 25°C at the top) may help to reduce the levels of vicinal diketones in lager beer produced by tower fermentation. However, if the fermenter is to be operated at high volumetric efficiencies the beer will require post fermentation treatment to reduce the level of vicinal diketones.

It has been claimed that it is possible to obtain a product

with a constant composition from a tower fermenter by careful control of the operating conditions (Klopper et alia, 1965). It is equally important however, that the yeast culture employed retains its original characteristics. Mutation may be a serious hazard in brewing and it is disquieting to read reports that this phenomenon has been the cause of problems in breweries operating the batch fermentations with strains of Saccharomyces uvarum.

To the author's knowledge there have been no reports of similar problems with strains of Saccharomyces cerevisiae.

A study of a brewery strain of Saccharomyces cerevisiae indicated that the culture was substantially unchanged after approximately 4 months continuous cultivation in the laboratory tower fermenter. Similar results were obtained in a study of the same yeast which had been cultivated in a commercial fermenter for a period of one year.

It has been suggested that brewing strains of Saccharomyces cerevisiae are genetically more stable than brewing strains of Saccharomyces uvarum (Hall, 1972). Certainly it is to be doubted whether mutation will be a problem in the tower fermentation of ale beer. In view of Thorne's findings (1968b; 1970) that Saccharomyces cerevisiae is susceptible to mutation in continuous culture the use of suitable strains of Saccharomyces cerevisiae for the production of lager beer should be investigated.

The work presented in the final section of this thesis represents an extension of the application of the tower fermenter. The results of experiments conducted with the laboratory tower fermenter indicated that the productivity of the system was higher than a batch or single stage homogeneous system (Bose and Ghose, 1973) for fermenting molasses but that the yield of alcohol was

lower. It was possible to ferment concentrated media (up to 20% w/v sugar) satisfactorily in the laboratory fermenter but the productivity was reduced fourfold compared with that achieved when fermenting media containing 10% w/v sugar.

By modification of the medium and the use of a higher temperature (28°C) it was possible to produce a liquor containing between 6.08 and 6.21 % w/v alcohol in 3.3 hours with 94% utilization of the fermentable carbohydrate. However, the productivity of the system was limited by the requirement for almost complete utilization of the fermentable material. Hospodka (1966) has suggested that the fermentation of the final residues of the sugar at alcohol concentrations above 7% by volume is uneconomical because of its slowness.

The production of alcohol from wheat mashes was studied by Yarovenko and Nakmanovich (1973) who found that fermentation rate adequately described by first order reaction kinetics of the type

$$\frac{dy}{dt} = k_1 (a - y)$$

where a = initial sugar concentration in the medium

y = amount of sugar fermented at time t

and thus a - y = residual sugar at time t

The constant k_1 is determined by

$$k_1 = \frac{2.303}{t} \log \frac{a}{a - y}$$

For wheat mashes the quantity of alcohol (P) produced at time (t) is described by

$$P = 0.622t\ a\ \ 1 - \exp\ (-k_1 t)$$

The kinetics of molasses fermentation to alcohol in the tower fermenter remain to be determined but the fermentation of brewer's wort in a tower fermenter approximates to two consecutive zero

order reactions (Greenshields and Smith, 1971).

The results which have been obtained indicate that the tower fermenter is a viable alternative to a multi stage homogeneous process which has been generally employed for the production of alcohol.

The future developments in tower production of alcohol may involve non-aseptic fermentation. It may be possible to ferment non-sterile mashes because the rapid rise in the alcohol concentration and the high liquid flow rates should prevent serious contaminations by bacteria. Indeed at one stage a bacterial infection was observed in the tower fermenter and was successfully eliminated by increasing the medium flow rate.

This is in contrast to the author's experience of beer fermentation which cannot be rescued once an infection has gained access to the tower.

In view of these observations it should be possible to design a process employing a tower fermenter for the production of ethanol. The fermentable substrate (say molasses) could be stored in a concentrated form and diluted in line to the fermenter. A flash pasteurization of the liquor should kill vegetative forms of undesirable micro-organisms and the conditions within the tower should hinder the development of spores which may be present in the medium.

Although the tower fermenter has been employed for the production of potable alcohol and vinegar charging wort (Greenshields and Smith, 1974) other forms of anaerobic fermentation have yet to be investigated. It seems likely that the production of organic solvents by fermentation may again become an important process. It may be that the heterogeneous mode of operation of the tubular

reactor can be exploited to produce acetone and butanol in a single stage process. These solvents are toxic to the microorganisms in concentration in excess of 1 to 2 % and the normal practice has been to employ a multistage process similar to that employed for alcohol production from molasses.

In conclusion it can be stated that the laboratory scale fermenter has proved a useful tool in the investigation of certain microbiological problems of anaerobic tower fermentation. However, the shortened form of the fermenter has presented some problems, mainly longitudinal mixing, and it is evident that the kinetics of tower fermentation processes will only be established at the pilot or commercial scale.

APPENDIX

TABLE A 1

CFCC 1. Giant Colony Characteristics

Isolate No.	Size	Edge	Surface	Elevation	Colour
Parent	16.8	Serrated	Smooth Matt	a	Cream
1	16.2	Serrated	Smooth Matt	a	Cream
2	19.2	"	" "	a	"
3	15.0	"	" "	a	"
4	16.7	"	" "	a	"
5	22.0	"	" "	a	"
6	22.5	"	" "	a	"
7	17.0	"	" "	a	"
8	17.0	"	" "	a	"
9	16.3	"	" "	a	"
10	18.5	"	" "	a	"
11	16.3	"	" "	a	"
12	11.0	Lobed	Gritty Matt	a	Brown
13	16.5	Serrated	Smooth Matt	a	Cream
14	16.0	"	" "	a	"
15	15.7	"	" "	a	"
16	17.0	"	" "	a	"
17	15.8	"	" "	a	"
18	18.8	"	" "	a	"
19	22.0	"	" "	a	"
20	17.5	"	" "	a	"
21	15.0	"	" "	a	"
22	16.7	"	" "	a	"
23	15.7	Lobed	" "	a	"
24	20.5	Serrated	" "	a	"

TABLE A1 - cont.

Isolate No.	Size	Edge	Surface	Elevation	Colour
25	21.0	Serrated	Smooth Matt	a	Cream
26	18.2	"	" "	a	"
27	18.3	"	" "	a	"
28	18.3	"	" "	a	"
29	16.0	"	" "	a	"
30	14.5	"	" "	a	"
31	15.5	"	" "	a	"
32	16.5	"	" "	a	"
33	26.0	"	" "	a	"
34	13.0	"	" "	b	"
35	18.0	"	" "	a	"
36	16.5	"	" "	a	"
37	17.7	"	" "	a	"
38	16.7	"	" "	a	"
39	18.3	"	" "	a	"
40	16.7	"	" "	a	"
41	17.5	"	" "	a	"
42	18.0	"	" "	a	"
43	23.2	"	" "	a	"
44	22.5	"	" "	a	"
45	22.3	"	" "	a	"
46	20.5	"	" "	a	"
47	20.5	"	" "	a	"
48	17.0	"	" "	a	"
49	14.0	"	" "	a	"
50	15.0	"	" "	a	"

Key to Elevation: graded a, b, c on visual examination

APPENDIX

TABLE A 2

CFCC 1: Characteristics of the isolates

Isolate No.	Cell Shape	Mean Cell Width (μ)	Flocculence Value (Sharp)	Spore Formation	Melibiose fermentation
Parent	n	5.6	93.1	++	-ve
1	n	5.8	114	+	-ve
2	n	5.7	91	+	-ve
3	n	5.7	100	++	-ve
4	n	5.6	98	++	-ve
5	n	6.2	103	++	-ve
6	n	5.6	92	++	-ve
7	n	6.0	96	++	-ve
8	n	6.2	116	++	-ve
9	n	6.5	114	++	-ve
10	n	5.6	94	++	-ve
11	n	6.1	102	++	-ve
12	n	5.8	121	++	-ve
13	n	5.9	123	++	-ve
14	n	5.7	121	++	-ve
15	n	5.2	122	++	-ve
16	n	5.8	112	++	-ve
17	n	5.8	102	++	-ve
18	n	6.0	114	+	-ve
19	n	5.8	110	++	-ve
20	n	5.6	104	++	-ve
21	n	5.6	102	++	-ve
22	n	6.8	110	++	-ve
23	n	5.9	100	++	-ve
24	n	5.4	89	++	-ve
25	n	5.9	99	+	-ve

TABLE A2 - continued

Isolate No.	Cell Shape	Mean cell width (μ)	Flocculence value (Sharp)	Spore Formation	Melibiose fermentation
26	n	6.0	93	+	-ve
27	n	6.1	97	++	-ve
28	n	5.7	102	+	-ve
29	n	5.7	99	++	-ve
30	n	5.3	111	++	-ve
31	n	5.7	99	+	-ve
32	n	5.1	111	+	-ve
33	n	5.7	87	++	-ve
34	n	5.5	101	++	-ve
35	n	5.5	70	+	-ve
36	n	5.6	88	++	-ve
37	n	5.8	84	++	-ve
38	n	5.8	91	+	-ve
39	n	6.2	102	++	-ve
40	n	6.1	106	+	-ve
41	n	5.8	98	++	-ve
42	n	5.9	118	++	-ve
43	n	6.4	118	+	-ve
44	n	5.2	108	+	-ve
45	n	5.6	94	+	-ve
46	n	6.0	121	++	-ve
47	n	6.4	104	++	-ve
48	n	5.7	116	+	-ve
49	n	5.0	113	+	-ve
50	n	6.0	114	+	-ve

Key n = normal
e = elongate
+ sparse
++ positive
0 negative
+ve positive
-ve negative

APPENDIX

TABLE A 3

CFCC 70: Giant colony characteristics

Isolate No.	Size	Edge	Surface	Elevation	Colour
Parent	17	Serrated	Smooth Matt	a	Cream
1	17	Serrated	Smooth Matt	a	Cream
2	21	"	" "	a	"
3	16	"	" "	a	"
4	16	"	" "	a	"
5	15	"	" "	a	"
6	18	"	" "	a	"
7	15	"	" "	a	"
8	13	"	" "	a	"
9	18	"	" "	a	"
10	–	–	– –	–	–
11	22	"	" "	a	"
12	18.5	"	" "	a	"
13	16.5	"	" "	a	"
14	17	"	" "	a	"
15	17.5	"	" "	a	"
16	14	"	" "	a	"
17	–	–	– –	–	–
18	14	"	" "	a	"
19	13	"	" "	a	"
20	13	"	" "	a	"
21	16	"	" "	a	"
22	14	"	" "	a	"
23	14	"	" "	a	"
24	–	–	– –	–	–
25	14	"	" "	a	"

TABLE A 3- continued

Isolate No.	Size	Edge	Surface	Elevation	Colour
26	14	Serrated	Smooth Matt	a	Cream
27	15	"	" "	a	"
28	-	-	- -	-	-
29	16	"	" "	a	"
30	17	"	" "	a	"
31	19	"	" "	a	"
32	-	-	- -	-	-
33	-	-	- -	-	-
34	17	"	" "	a	"
35	14	"	" "	a	"
36	17	"	" "	a	"
37	17.5	"	" "	a	"
38	16	"	" "	a	"
39	17	"	" "	a	"
40	16	"	" "	a	"
41	18.5	"	" "	a	"
42	15	"	" "	a	"
43	17.5	"	" "	a	"
44	12.5	"	" "	a	"
45	14	"	" "	a	"
46	12.5	"	" "	a	"
47	16	"	" "	a	"
48	16	"	" "	a	"
49	15	"	" "	a	"
50	18	"	" "	a	"

Key - Elevation graded a, b, c on visual examination

APPENDIX

TABLE A 4

CFCC 70: Characteristics of the isolates

Isolate No.	Cell Shape	Mean Cell width (μ)	Flocculence Value (Sharp)	Spore Formation	Melibiose Fermentation
Parent	n	5.2	127.6	++	-ve
1	n	5.3	120	0	-ve
2	n	5.5	110	0	-ve
3	n	5.6	120	+	-ve
4	n	4.7	118	+	-ve
5	n	5.1	120	++	-ve
6	n	4.5	111	++	-ve
7	n	4.8	113	0	-ve
8	n	4.3	110	0	-ve
9	n	3.5	117	++	-ve
10	n	4.7	97	0	-ve
11	n	4.6	115	++	-ve
12	n	5.5	119	++	-ve
13	n	5.6	112	++	-ve
14	n	5.3	105	++	-ve
15	n	5.2	108	++	-ve
16	n	4.6	112	0	-ve
17	n	5.3	102	++	-ve
18	n	4.8	114	0	-ve
19	n	5.2	115	0	-ve
20	n	5.3	107	+	-ve
21	n	5.6	130	+	-ve
22	n	5.6	124	+	-ve
23	n	5.0	124	+	-ve
24	n	5.4	122	+	-ve
25	n	5.0	112	+	-ve

n = normal
e = elongate

+ sparse
++ positive
0 negative

+ve positive
-ve negative

TABLEA 4- continued

Isolate No.	Cell Shape	Mean Cell width (μ)	Flocculence Value (Sharp)	Spore Formation	Melibiose ferment-ation
26	n	5.2	118	+	-ve
27	n	5.5	122	+	-ve
28	n	6.2	124	+	-ve
29	n	5.6	99	++	-ve
30	n	5.4	135	+	-ve
31	n	5.1	99	+	-ve
32	n	5.4	134	+	-ve
33	n	5.5	110	+	-ve
34	n	5.8	110	+	-ve
35	n	5.2	119	++	-ve
36	n	5.1	122	++	-ve
37	n	4.9	128	++	-ve
38	n	5.1	113	+	-ve
39	n	5.0	120	+	-ve
40	n	5.2	118	+	-ve
41	n	5.0	116	+	-ve
42	n	5.1	124	++	-ve
43	n	5.2	125	++	-ve
44	n	6.1	122	+	-ve
45	n	6.2	127	+	-ve
46	n	5.0	125	+	-ve
47	n	5.2	104	+	-ve
48	n	5.2	116	+	-ve
49	n	4.9	113	+	-ve
50	n	4.8	114	++	-ve

TABLE A.4- continued

Isolate No.	Fermentation Rate	Yeast Yield (g)	Final Attenuation
Parent	0.00385	46.5	97.6
1	0.0040	44	96.6
2	0.0038	45	97.2
3	0.0036	47	97.1
4	0.0035	43	95.7
5	0.0036	42	97.1
6	0.0042	42	95.8
7	0.0040	43	98.6
8	0.0040	44	98.6
9	0.0039	43	95.7
10	0.0040	45	95.8
11	0.0042	42	96.4
12	0.0043	40	92.5
13	0.0040	45	94.5
14	0.0038	42	93.7
15	0.0037	44	91.8
16	0.0036	41	98.4
17	0.0033	42	98.5
18	0.0033	55	96.0
19	0.0041	49	97.3
29	0.0035	52	97.3
21	0.0036	50	96.5
22	0.0035	52	96.5
23	0.0040	54	96.0
24	0.0044	51	97.8
25	0.0042	49	97.8

TABLE A 4- continued

Isolate No.	Fermentation Rate	Yeast Yield (g)	Final Attenuation
26	0.0034	50	97.8
27	0.0038	53	96.7
28	0.0037	52	96.5
29	0.0034	52	96.6
30	0.0034	51	95.3
31	0.0038	50	95.3
32	0.0037	52	97.3
33	0.0035	51	96.5
34	0.0039	54	96.0
35	0.0035	55	94.7
36	0.0033	53	97.4
37	0.0034	49	96.1
38	0.0038	51	96.1
39	0.0035	52	97.3
40	0.0035	49	97.4
41	0.0037	49	96.1
42	0.0038	42	96.6
43	0.0038	42	94.0
44	0.0039	42	94.8
45	0.0034	44	93.6
46	0.0040	45	94.9
47	0.0042	43	93.5
48	0.0040	43	96.6
49	0.0041	42	96.9
50	0.0041	40	96.1

APPENDIX

Information relating to Graphs 14 - Table A 5.

Graph 14 Yeast CFCC 39

Volumetric Efficiency	Temperature °C	Wort O.G.	Beer P.G.	Beer total vdk. mg/litre	Beer pH
1.0	20	1.050	1.0113	0.225	-
			1.0113	0.260	-
			1.009	0.275	-
1.7			1.0112	0.330	4.3
			1.0113	0.490	-
			1.0115	0.380	4.15
			1.0100	0.320	4.04
2.6			1.0108	0.455	4.7
			1.0115	0.665	4.15

Graph 15 Yeast CFCC 83 - Table A.6

0.5	20	1.050	1.0122	0.107	3.98
			1.0125	0.105	3.95
			1.0117	0.110	3.95
			1.0127	0.095	-
0.9			1.0122	0.290	3.75
			1.0120	0.265	3.85
			1.0122	0.195	3.85
1.35			1.0125	0.330	3.80
			1.0123	0.325	3.95
1.6			1.0132	0.430	3.85
			1.013	0.492	3.80
			1.013	0.435	3.80
2.25			1.0127	0.600	-
			1.0130	0.543	4.2

Graph 16 Yeast CFCC 83 - Table A 7.

Volumetric Efficiency	Temperature °C	Wort O.G.	Beer P.G.	Beer total vdk. mg/litre	Beer pH
1.2	15	1.050	1.0103	0.230	-
			1.0098	0.325	4.15
			1.0103	0.320	4.00
			1.0110	0.290	4.25
1.45			1.0105	0.325	-
			1.010	0.290	4.1
			1.0098	0.420	4.15
			1.0105	0.500	4.1
			1.0105	0.485	4.2
1.8			1.0110	0.290	4.1
			1.0105	0.460	-
2.5			1.0110	0.820	-
			1.0100	0.610	4.3

Appendix ix

Table A9

Molasses medium. Run 1. Experiment 1.

Volumetric efficiency	Medium sugar conc. g/100 ml.	Fermented liquor	
		residual sugar g/100 ml.	Alcohol conc. g/100 ml.
0.9	9.2	0.713	3.78
	9.2	0.750	4.32
	10.05	0.876	4.02
1.46	10.05	0.766	4.14
	10.05	0.956	4.20
	10.05	0.884	3.90
	9.92	0.893	4.51
	9.92	0.845	3.90
	9.92	0.869	3.90
2.9	9.93	0.895	3.60
	9.93	0.818	3.72
	10.10	0.893	3.72
	10.34	0.849	3.60
	10.34	0.847	3.72
	10.34	0.914	3.72
3.5	10.00	0.910	3.72
	10.00	0.958	3.78
	10.00	0.875	4.02
	10.07	0.813	3.90
	10.07	0.793	3.48
5.4	10.02	1.08	3.78
	10.02	1.15	3.72
	10.02	1.01	3.37

Appendix

Table A10

Molasses medium. Run 2. Experiment 1.

Volumetric efficiency	Medium sugar conc. g/100 ml.	Fermented liquor	
		residual sugar g/100 ml.	Alcohol conc. g/100 ml.
1.20	10.55	0.920	3.6
	10.55	0.960	3.6
	10.17	0.778	3.96
	10.17	0.778	4.08
1.55	10.00	1.080	3.84
	10.00	0.930	3.84
	10.00	0.792	3.84
	10.00	0.830	3.84
2.5	10.175	1.017	3.84
	10.175	0.875	3.84
	10.175	0.865	3.84
	10.175	0.822	3.84
	10.175	0.934	3.90
3.2	10.21	1.12	3.96
	10.21	1.208	3.96
	10.21	1.10	3.96
	10.21	1.13	3.90
	10.21	1.08	3.90

Appendix

Table A 11

Molasses medium. Run 3. Experiment II.

Volumetric efficiency	Medium sugar conc. g/100 ml.	Fermented Liquor	
		residual sugar g/100 ml.	Alcohol conc. g/100 ml.
2.5	14.65	1.805	5.13
	14.65	1.718	5.82
	14.65	1.350	6.02
	15.10	1.395	5.64
2.95	14.65	1.525	5.76
	15.77	1.549	5.95
	15.77	2.080	6.08
	15.21	1.845	5.95
	15.21	2.125	5.82
	15.21	1.910	5.51
2.0	17.70	1.847	7.28
	17.70	1.697	7.01
	17.70	1.692	7.35
	17.70	1.807	7.22
	17.70	1.865	7.01
1.1	20.00	2.23	8.25
	20.00	2.53	7.35
	20.00	2.51	7.76
	20.00	2.48	7.69

Appendix

Table A 12

Molasses medium. Run 3. Experiment II

Day	Medium conc. g/100 ml.	Volumetric Efficiency	Medium N. g/100 ml.	Beer N. g/100 ml.	Uptake of N. (%)
18	10.00	0.9	0.355	0.308	13.2
19			0.355	0.316	10.9
21			0.353	0.324	8.2
22		1.65	0.353	0.344	2.54
24			0.359	0.345	3.89
25			0.359	0.347	3.34
28		1.4	0.359	0.353	1.67
29			0.359	0.347	3.34
30			0.359	0.344	4.17
31		1.2	0.361	0.336	6.92
33			0.354	0.340	3.95
35			0.354	0.339	4.23
36		1.5	0.380	0.333	12.36
38			0.380	0.341	10.26
39		1.9	0.342	0.337	1.46
39			0.342	0.333	2.63
40			0.342	0.337	1.46
40		2.9	0.342	0.337	1.46
41			0.388	0.347	10.56
42			0.388	0.349	10.05
43			0.361	0.345	4.43
44			0.337	0.360	- 6.82
44		3.6	0.337	0.366	- 8.60
45			0.337	0.361	- 7.12
45		3.3	0.354	0.355	- 0.28
46			0.354	0.347	1.97
46			0.354	0.347	1.97
46		3.6	0.349	0.349	0
47			0.349	0.347	0.57
48		4.0	0.353	0.344	2.55
49			0.353	0.393	-11.32
50			0.346	0.344	0.58
50		5.4	0.347	0.343	1.1
52			0.356	0.349	1.97

Table A 12 continued

Day	Medium conc. g/100 ml.	Volumetric Efficiency	Medium N. g/100 ml.	Beer N. g/100 ml	Uptake of N (%)
52	10.00	5.4	0.356	0.354	0.57
53	15.00	2.5	0.476	0.334	29.85
54		2.5	0.481	0.488	- 1.46
55			0.481	0.473	1.7
56			0.469	0.488	- 4.05
57			0.469	0.480	- 2.35
57			0.469	0.475	- 1.28
58		2.94	0.469	0.486	- 3.625
58			0.470	0.487	- 3.62
59			0.470	0.473	- 0.64
59		2.8	0.460	0.470	- 2.13
60			0.460	0.470	- 2.13
60			0.460	0.473	- 2.11
64	17.00	1.2	0.546	0.533	2.38
65		2.0	0.546	0.543	0.55
66			0.578	0.562	2.67
68			0.576	0.588	- 2.02
68		1.2	0.576	0.583	- 1.22
70	20.00	1.2	0.613	0.589	6.1
71			0.613	0.628	- 2.43
71			0.613	0.614	- 0.16
72		1.1	0.613	0.650	- 6.05
72			0.613	0.677	-10.05
73			0.613	0.658	- 7.35

Appendix

Table A 13

Molasses - sucrose medium. Run 4. Experiment III

Volumetric Efficiency	Medium sugar conc. g/100 ml.	Fermented liquor	
		residual sugar g/100 ml.	Alcohol conc. g/100 ml.
1.2	15.25	0.874	5.89
	15.25	0.898	5.89
	15.25	0.870	6.21
	15.25	0.817	6.21
2.25	13.95	0.970	6.21
	13.95	0.963	6.21
	13.95	0.925	6.08
2.8	14.15	1.173	6.08
	14.15	0.923	6.28
	14.50	1.03	6.28
	14.50	1.05	6.15
	14.50	1.07	6.21
7.2	14.90	2.04	5.82
	14.90	2.03	5.82
	14.90	2.12	5.82
	14.90	2.11	5.82
	14.90	1.95	5.95

Appendix

Table A 14

Molasses - sucrose medium. Run 4. Experiment II.

Volumetric Efficiency	Temp. °C	Medium sugar conc. g/100 ml.	Fermented liquor	
			Residual sugar g/100 ml	Alcohol conc. g/100 ml
7.2	20	14.9	2.125	-
		14.9	2.04	5.82
		14.9	2.035	5.82
		14.9	2.12	5.82
		14.9	2.11	5.82
		14.9	2.25	5.82
		14.85	2.15	5.89
	28	15.15	1.51	6.21
		15.15	1.495	6.08
		15.15	1.385	6.08
		15.15	1.385	6.15
		15.15	1.415	6.21

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