AN INVESTIGATION OF FACTORS INFLUENCING THE PRODUCTION OF 2, 3, BUTANEDIONE BY SACCHAROMYCES CARLSBERGENSIS

by

Francis Lowell Palmer

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TABLE OF CONTENTS

INDEX OF FIGURES

INTRODUCTION

The general trend toward the production of lightly hopped, high adjunct ratio beers has tended to uncover certain flavour abnormalities which were masked by the more heavily hopped and flavoured beers of earlier years. These flavours and aromas are due primarily to the presence of esters, aldehydes and higher alcohols formed as by-products in the normal fermentation cycle and have become accepted as contributory factors to the characteristic bouquet of the product. In recent years, however, a buttery, honey-like flavour attributed to the presence of 2, 3, butanedione or diacetyl has become particularly noticeable. In lightly hopped beers this character usually is considered to be undesirable.

This investigation is concerned with a study of the factors which may influence the production of 2, 3, butanedione in full-scale brewery fermentations.

HISTORICAL

In general, three theories have been proposed to account for diacetyl production in brewery fermentations. The first theory proposes that diacetyl is produced by bacterial contaminants present in brewery yeast cultures. In a recent investigation, Unterkofler and Hickey (10) examined 174 strains of anaerobic bacteria belonging to 64 species and found that over 70 percent of them produced acetoin which on oxidation becomes diacetyl. Shimwell (9) has characterized one of the common diacetyl-producing bacterial contaminants in brewery yeast cultures as a member of the lactic streptococcus group. This species grows in the form of spheres occurring singly, in pairs, tetrads, clumps or chains and has an optimum temperature for growth between 21° C and 25° C. Further, it is described as homofermentative; producing acid from glucose, fructose, maltose and sucrose but not from pentoses or from salacin. In hopped wort or beer, growth is accompanied by the production of diacetyl.

Earlier investigators classified this organism in the genus Sarcina thus leading to the use of the term "sarcina sickness" to describe the characteristic diacetyl odour and taste found in certain beers. A review of the literature indicates the species has been named variously by different investigators to include the following: Streptococcus damnosus (Claisen, Shimwell and Kirkpatrick), Pediococcus damnosus (Claisen), Pediococcus perniciosus (Claisen), Pediococcus cerevisiae (Balke) and Pediococcus sarcinaeformis (Reichard).

A second theory to account for the presence of diacetyl in beer was proposed by Czarnecki and Engel (3). It was based on the assumption that yeast variants within the "normal" yeast population were responsible for the production of diacetyl. In this respect, three types of variants are recognized: the haploids, produced by sporulation; the mutants which arise from gene damage or alteration, and the "petites" which have undergone cytoplasmic alterations during budding that resulted in a respiratory deficiency partly due to the loss of succinic dehydrogenase and of cytochrome oxidase.

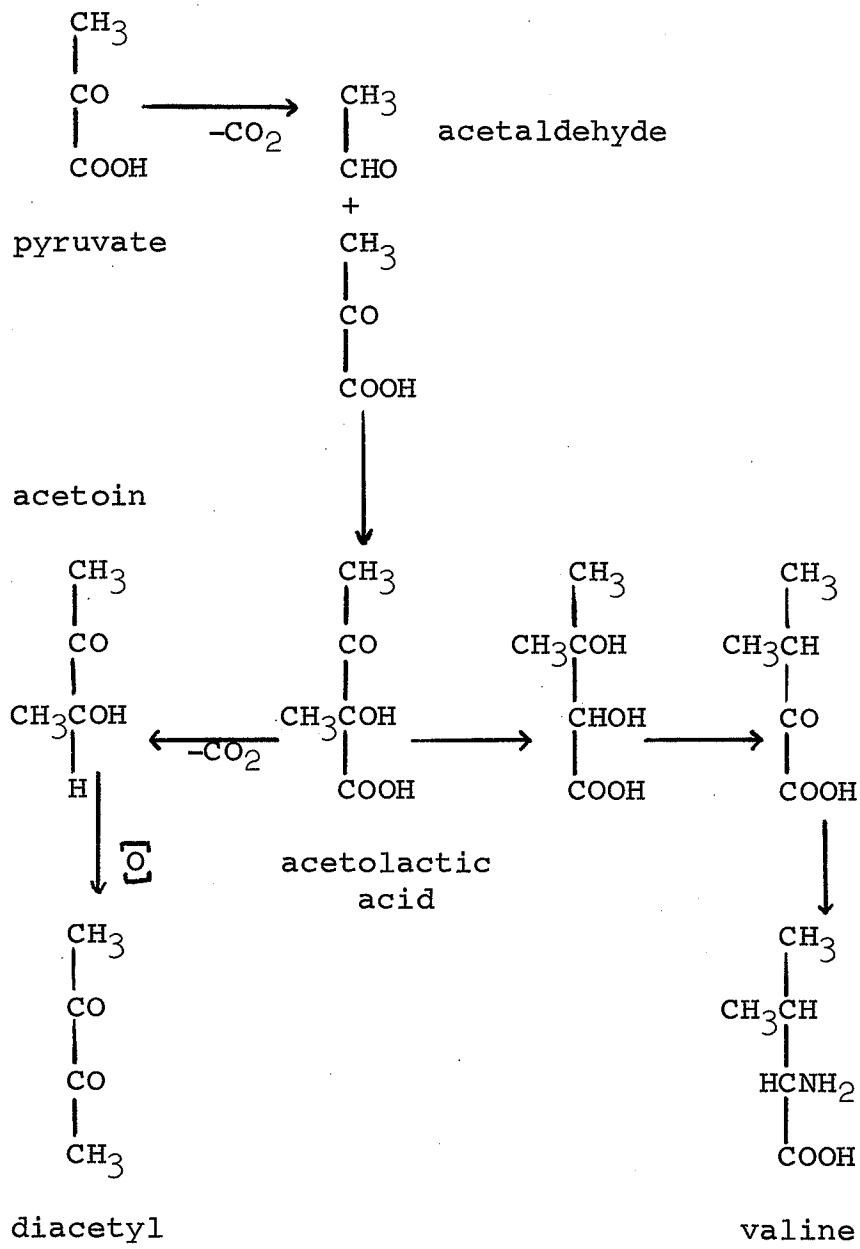
Generally, brewers' yeast strains have lost the ability to sporulate and sporulation is very rarely obtained even under the most favourable conditions. Furthermore those spores that are produced occasionally are seldom viable. In the light of this evidence, it is very unlikely that a diacetyl-producing haploid strain could develop through successive generations.

In an investigation of respiratory-deficient yeasts, Czarnecki and Engel (3) plated various dilutions of different brewers' yeast strains on Wallerstein's Nutrient medium containing brom-cresol green as an indicator. On this medium colonies of "normal" yeast develop a whitish to pale green color. In addition to colonies of normal color, three other colony types were observed. First, variants which were unable to reduce the brom-cresol green or at best could only reduce the indicator to a minor extent. These were designated as "verdants". Second, colonies with variegated light and dark green sectors were designated as "variegated". Third, colonies referred to as "petites" developed as white colonies with a dark central area.

Pure cultures of each of the three variant forms were isolated and used as inocula in fermentations of brewery wort. In all cases, abnormal beers with a spicy or diacetyl character resulted. Thus Czarnecki and Engel (3) concluded that "normal" brewery yeast may be contaminated by respiratory deficient yeasts and that these strains were responsible for the production of diacetyl in the fermentation.

A third theory to account for the presence of diacetyl was proposed by Owades, Maresca and Rubin (8) who presented evidence to show that diacetyl is produced by "normal" yeast as a by-product of its synthesis of valine. Their investigation showed that the addition of 200 p.p.m. of valine to a fermenting wort markedly depressed diacetyl production. The relationship between diacetyl formation and the synthesis of valine as suggested by Owades et al (8) is shown in Fig. 1. In this series of reactions a condensation of pyruvic acid and acetaldehyde leads to the formation of acetolactic acid which serves as the precursor for either valine or

Fig. 1. A proposed mechanism for the production of diacetyl according to Owades et al.



diacetyl. The possibility that the addition of other amino acid or nitrogen sources to the wort would have the same depressive effect on diacetyl formation as valine was also investigated. Several individual amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, methionine, lysine, threonine and tryptophane) were added at the rate of 500 p.p.m. In no case did any of the amino acid additives depress diacetyl formation to the extent observed for valine. Similar negative results were obtained when ammonium sulphate, yeast extract or casein hydrolysate were substituted for valine.

An experimental series was carried out to determine whether diacetyl production by the yeast populations used in this brewery (Lucky Lager Breweries, Bellingham, B.C.) could be attributed to the operation of any of the suggested schemes.

MATERIALS AND METHODS

Initially, it was considered necessary to examine the stock yeast cultures used in this brewery to determine if bacterial contaminants that might be responsible for diacetyl production were present. In addition to the examination of stock cultures, samples from actively fermenting wort were plated on Wallerstein's Differential medium. This medium contains actidione which prevents yeast development but permits the growth of those bacteria likely to be contaminant in the culture. Replicate sets of plated samples were incubated at 25° C aerobically and anaerobically for ten days.

An examination of yeast stock cultures for the presence of respiratory deficient strains was carried out by plating on Wallerstein's Nutrient medium as described by Czarnecki and Engel (3).

These surveys showed no evidence of either bacterial contaminants or respiratory deficient strains of yeast in any of the cultures examined.

Following the initial surveys, the investigation was divided into three parts. Part I was concerned with a determination of the rate at which diacetyl is produced during a normal fermentation cycle. Part II of the study was divided into two sections. Section A was designed to determine the effect of above-normal temperatures on diacetyl production while Section B was intended to show whether successive fermentations at above-normal temperatures would affect the pattern of yeast metabolism and thereby influence the levels of diacetyl subsequently produced. Part III of the study examined the effect of phosphoric acid washing practices on levels of diacetyl produced in fermentation by reclaimed cultures. While each of these parts is reported separately in the thesis, the analysis method for diacetyl determination is common to all parts and is presented herewith. The method of analysis used is basically the same as that reported by West *et al* (11). For each determination 250 ml of decarbonated beer was distilled in an atmosphere of CO_2 into 1.5 ml of

hydroxylamine hydrochloride (1 M). Forty five ml of distillate was collected and reduced to 18 ml on a water bath. This treatment evaporated the ethanol and concentrated the dimethylglyoxime. Titration followed with bromine water using a starch indicator. Nickel chloride was then added to form the red-colored tetravalent nickel dimethylglyoxime. Intensity of color in samples was determined colorimetrically at 430 mμ and compared with color intensities produced by known concentrations of dimethyl glyoxime. From the comparison of colorimetric readings a calculation of the diacetyl concentration in the sample was made. The total time elapsed for each analysis was three hours.

The term "available extract" used in the brewing industry means the amount of fermentable carbohydrate present as measured hygrometrically on the Plato Scale.

PART I

A DETERMINATION OF THE RATE OF DIACETYL PRODUCTION DURING A NORMAL FERMENTATION CYCLE

METHODS

Samples used in this part of the study were drawn from typical fermentation patterns as they develop in standard brewing practice. The temperature of the wort at time of inoculation was 50° F (10° C); temperature controls were set to permit a maximum temperature during fermentation of 56° F (13.3° C).

Samples were collected at daily intervals from the fermentation vessels and analyzed for diacetyl. Typical results of these analyses are presented graphically in Figs. 2, 3, and 4. The graphs indicate a possible temperature range from 44° F (6.7° C) to 68° F (20° C). The Plato scale indicates the extent of available extract from 13 to 1° P. Possible levels of diacetyl range from 0.05 to 0.65 p.p.m. Total elapsed time in

the fermenter was eight days.

RESULTS AND DISCUSSION

Patterns followed practically the same course in each of the fermentations. The diacetyl level increased rapidly, the highest level being coincident with maximum fermentation activity and when most of the available extract had been utilized. Available extract from an initial level of 11.8° P decreased to between 3 and 4° P. It should be noted that the maximum level of diacetyl is reached at this point. For reasons which are still obscure the level of diacetyl begins to decrease beyond this point in the fermentation. One explanation that might be proposed to account for this observation is that the yeast population in changing from a phase of maximum metabolic activity to essentially a resting condition begins to utilize diacetyl by a reversal of the mechanism through which it was formed. The possibility that diacetyl could be utilized beyond the formation of acetoin appears unlikely according to

Fig. 2. The rate of diacetyl production in a normal wort fermentation cycle, 50°F.(10°C.) to 56°F.(13.3°C.)

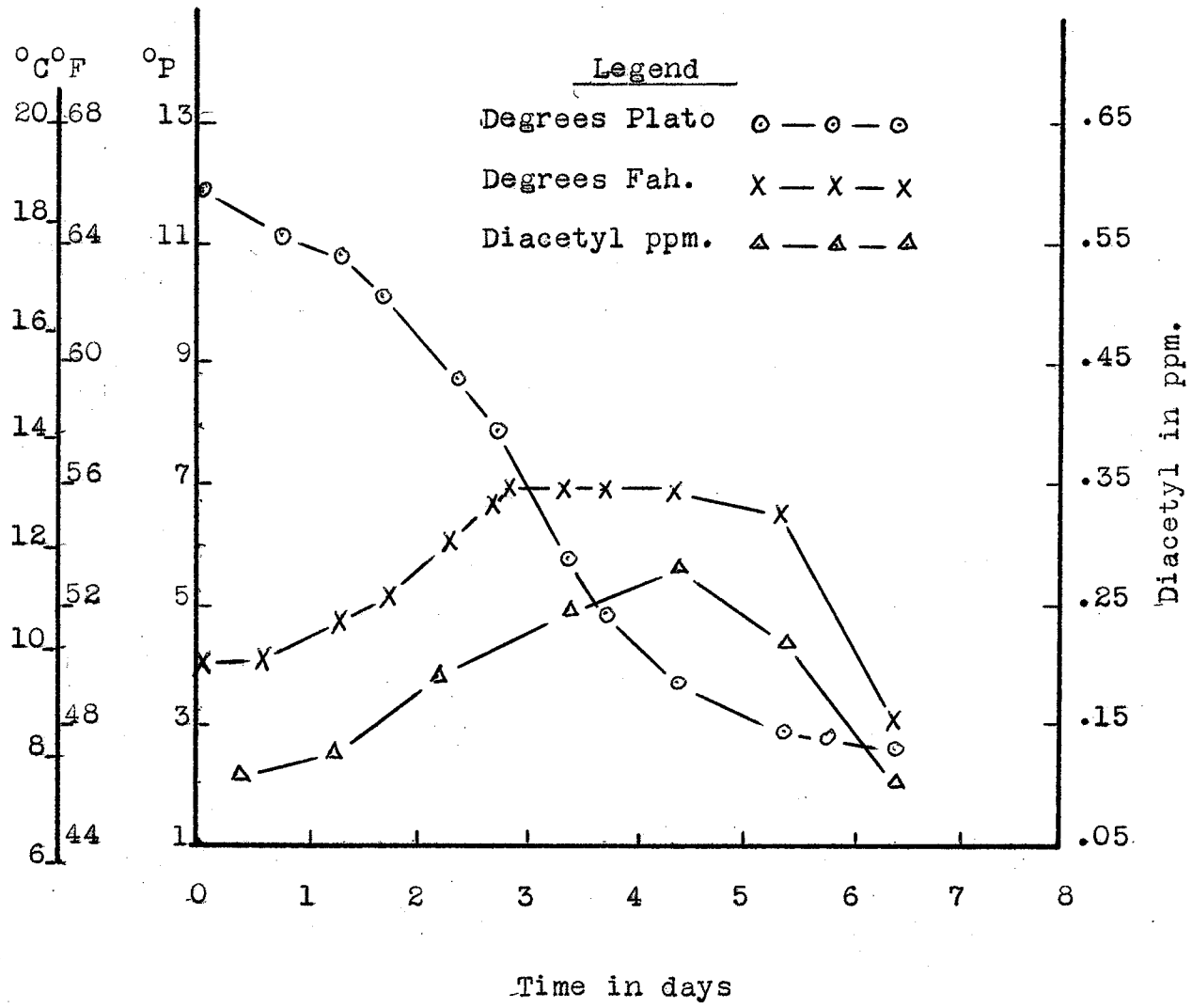


Fig. 3. The rate of diacetyl production in a normal wort fermentation cycle, 50°F.(10°C) to 56°F.(13.3°C)

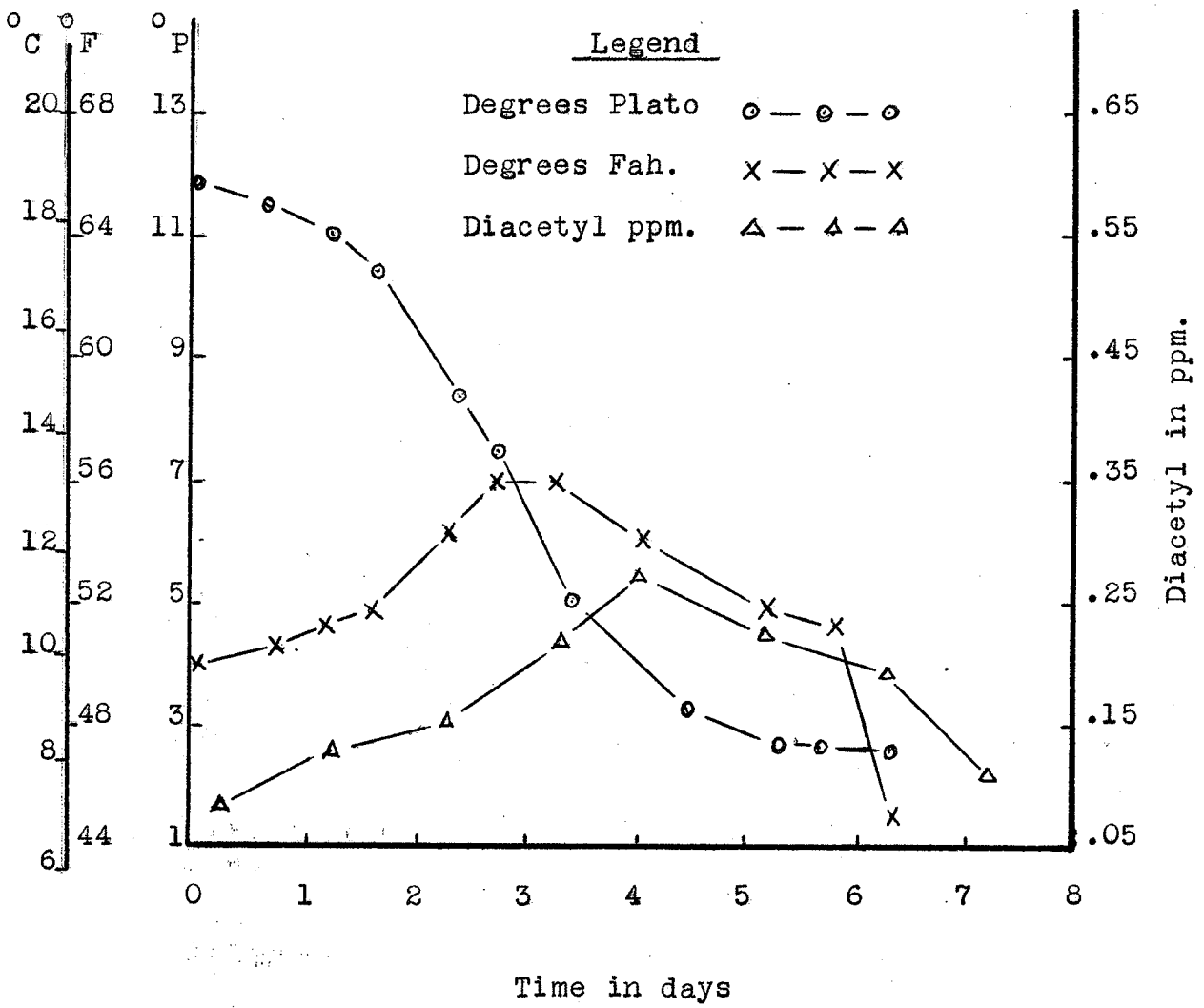
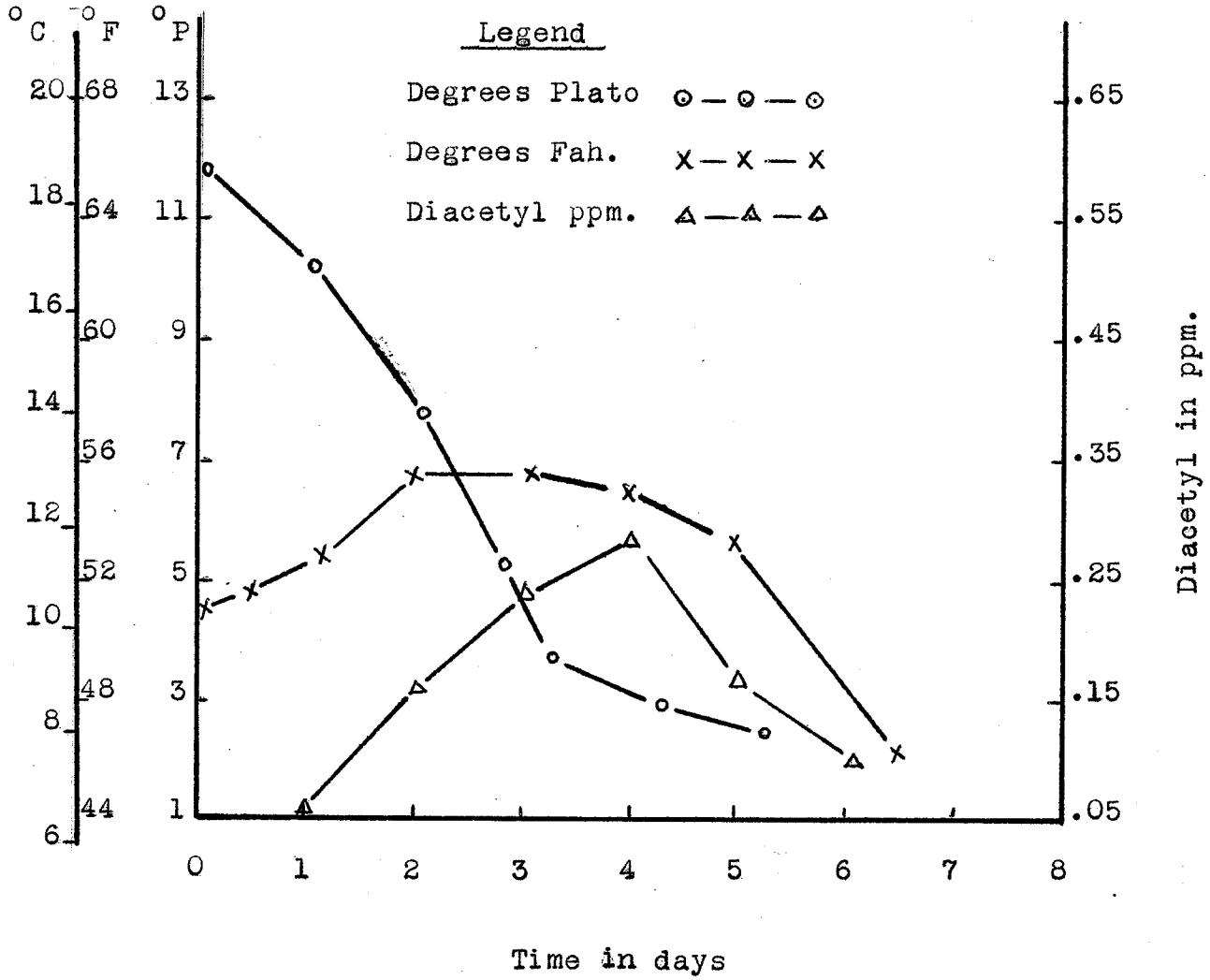


Fig. 4. The rate of diacetyl production in a normal wort fermentation cycle, 50 F.(10 C) to 56 F.(13.3 C.)



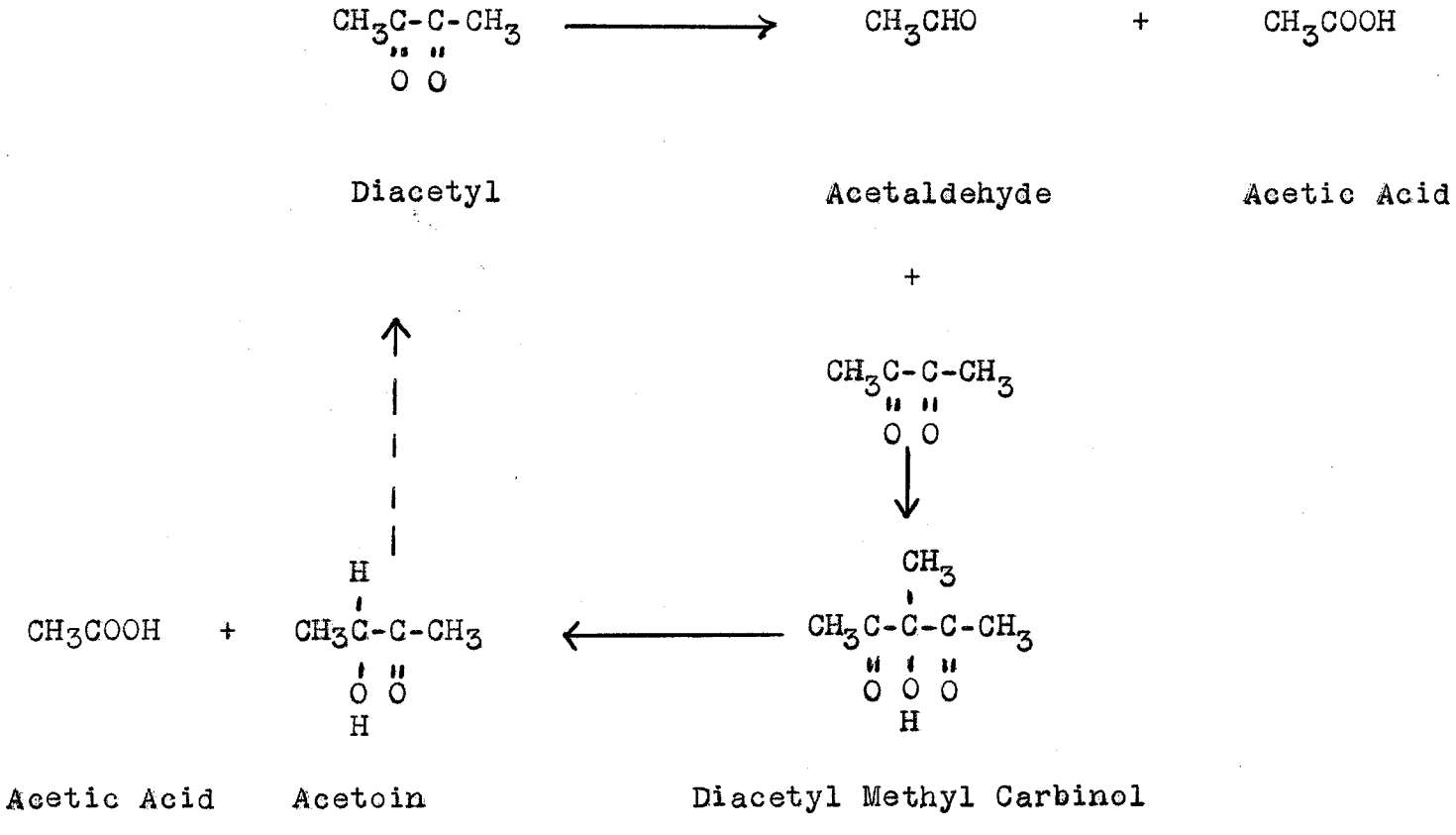
the scheme suggested by Owades *et al* (8) since this would involve a carboxylation to form acetolactic acid. The probability for this step in the sequence appears to be small.

Burger, Glenester and Becker (2) have reported that diacetyl levels in finished beer were lowered by the addition of fresh yeast. This finding was supported by Owades *et al* (8). Again, the mechanism by which the removal of diacetyl is accomplished is not known. On the other hand, Green (4) found a diacetyl mutase capable of converting diacetyl to acetoin and acetic acid according to the following equation:

$$2\ CH_3.CO.CO.CH_3 + 2\ H_2O \longrightarrow CH_3.CHOH.CO.CH_3 + 2\ CH_3COOH$$

Juni (5) proposed a diacetyl cycle through which one mole of diacetyl is split into two moles of acetic acid with α-methylcarbinol as the intermediate product. This scheme is illustrated in Fig. 5. Whether either of these mechanisms is operative in the latter stage of a wort fermentation is unknown.

Fig. 5. Proposed mechanism for the removal of diacetyl according to Juni.



PART II

GENERAL CONSIDERATIONS

The origin of lager yeast as represented by Saccharomyces carlsbergensis has been a subject for speculation for many years. Many unsuccessful attempts have been made to locate representatives of this type in a natural environment (7). It is now generally accepted that present day species are either mutants or strains that have undergone phenotypic modifications and have become acclimatized through many generations to a low temperature environment. It is also a general belief that continued fermentations at higher temperatures result in a product that has different qualities from that produced at the normal low temperature range.

Some evidence tends to support this belief. Kleber and Hoffman (6) have indicated that a reduction of enzyme capacity was concomitant with increased temperature maintained for successive generations during the reproductive stage of the cells.

Amaha and Takeuchi (1) demonstrated that vitamin requirements of yeast at low temperatures were much more exacting than at the higher range. These differences in yeast behaviour tend to support the concept that the nature and level of fermentation by-products may be affected by the temperature range maintained during the fermentation cycle.

PART II A

THE EFFECT OF ABOVE NORMAL TEMPERATURE ON THE RATE OF DIACETYL PRODUCTION IN WORT FERMENTATION

METHODS

Three experimental brews were started at an initial temperature of 54° F (12.2° C) and temperature controls set to provide a maximum of 60° F (15.6° C) as opposed to the normal cycle of 50 (10° C) to 56 F (13.3° C).

Again, samples were taken daily and diacetyl levels were determined as described previously.

RESULTS AND DISCUSSION

Diacetyl levels in the fermentation patterns are presented in Figs. 6, 7, and 8. No significant difference in final diacetyl levels was apparent in comparison to those obtained during a normal fermentation cycle.

Due to the accelerated fermentation, peak diacetyl levels were reached earlier than in the normal cycle but again coincided with the period of maximum yeast activity and when the fermentable extract had been reduced to between 3 and 4° Plato.

Fig. 6. The rate of diacetyl production in a normal wort fermentation cycle, 54 F.(12.2 C.) to 60 F.(15.6 C)

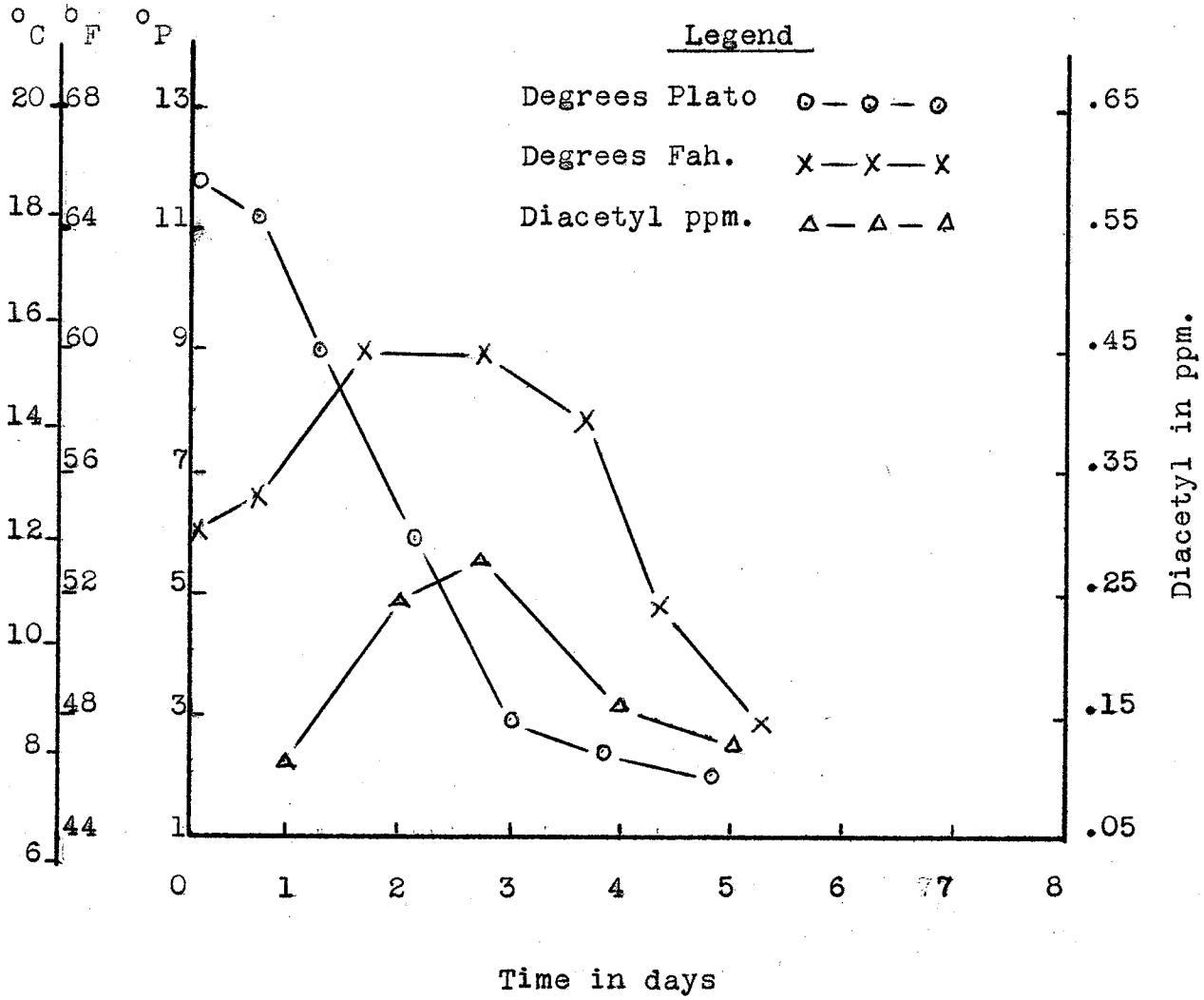


Fig. 7. The rate of diacetyl production in a normal wort fermentation cycle, 54 F. (12.2 C.) to 60 F. (15.6 C.)

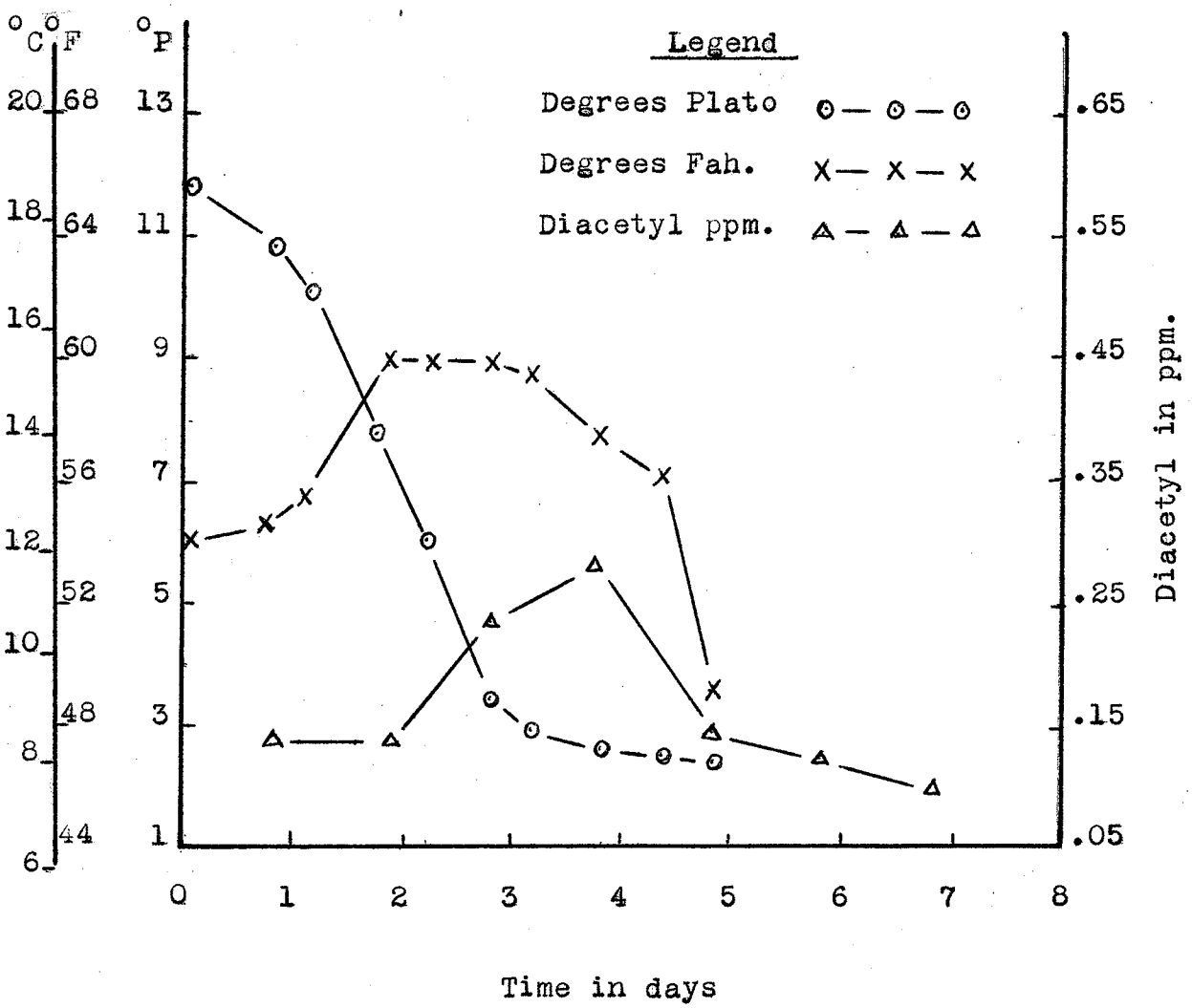
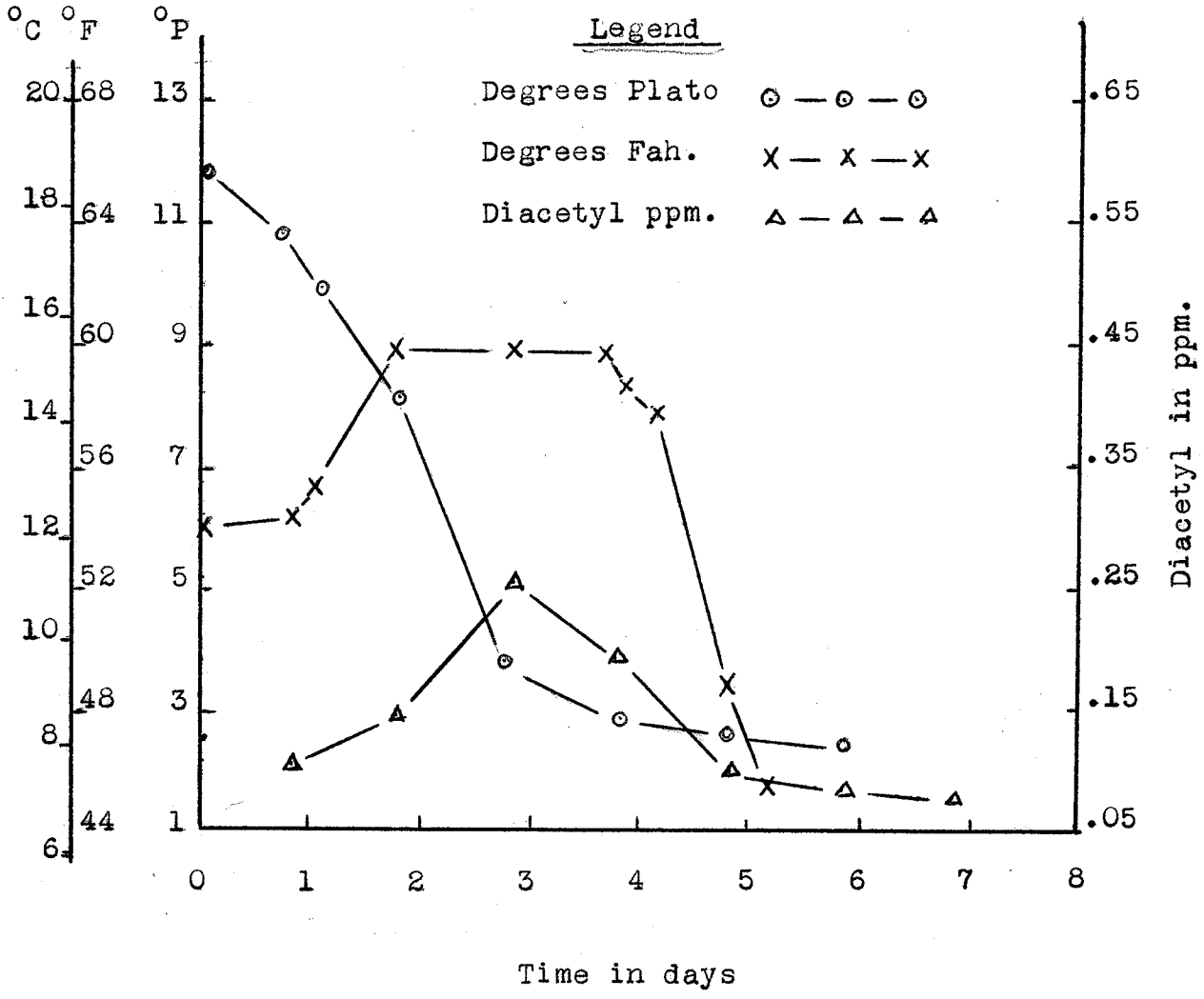


Fig. 8. The rate of diacetyl production in a normal wort fermentation cycle, 54 F.(12.2 C.) to 60 F.(15.6 C.)



PART II B

THE EFFECT OF ABOVE-NORMAL TEMPERATURE IN SUCCESSIVE FERMENTATIONS ON THE LEVELS OF DIACETYL PRODUCED

METHODS

A series of successive brews were fermented at 60 F (15.6° C). This temperature was maintained throughout the complete cycle. Yeast was reclaimed from each brew and used in the succeeding one. Diacetyl determinations were made on each brew at the end of the fermentation.

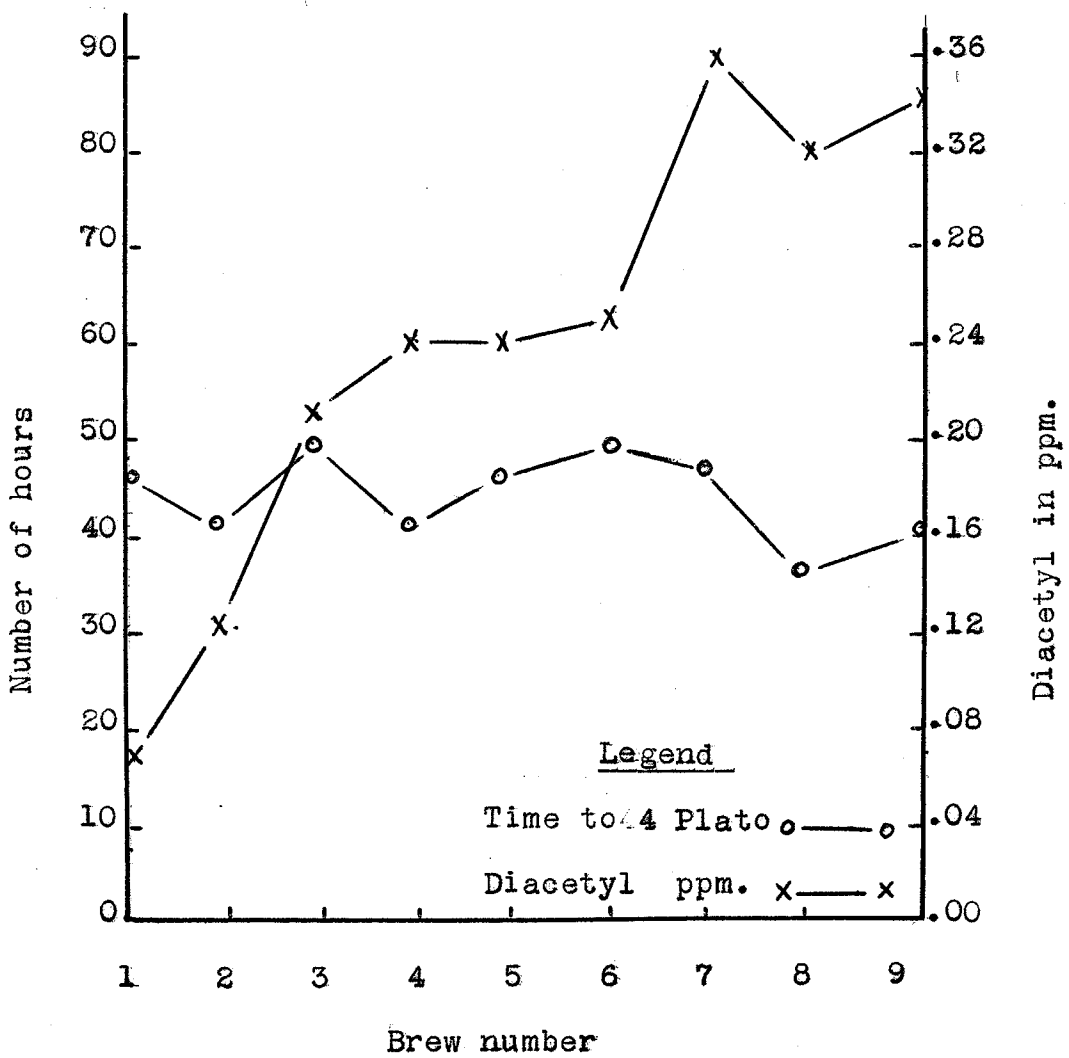
RESULTS AND DISCUSSION

Results obtained from the series of nine brews are presented in Fig. 9. Fermentations were very active as indicated by the rapid decrease in fermentable extract. Time required for the fermentable extract to drop to 4° P was between 35 and 50 hours as compared to that in the normal brews of 85 to 100 hours.

Diacetyl values started at 0.07 p.p.m. and in subsequent brews reached a maximum level of 0.36 p.p.m. at the end of the seventh cycle. Since a level of 0.25 p.p.m. of diacetyl has an unfavourable effect on the organoleptic properties of beer, this fermentation series with respect to diacetyl determination was discontinued.

It would appear that a temperature of 60 F (15.6° C) maintained for successive generations during the reproductive stage of a lager yeast initiates some change in metabolism leading to higher levels of diacetyl production.

Fig. 9. Diacetyl production in successive fermentations at 60°F.(15.6°C.)



PART III

AN INVESTIGATION OF PHOSPHORIC ACID WASHING PRACTICES ON DIACETYL PRODUCTION BY RECLAIMED YEAST

GENERAL CONSIDERATIONS

Many brewers make use of a phosphoric acid wash treatment of reclaimed yeast as a regular procedure in their operations. The acid is added to the yeast slurry in sufficient quantity to produce a pH of 2.1. The wash is maintained in continuous motion by means of a mixer for a period of four hours. The purpose of the wash is two fold: it destroys or severely inhibits various diacetyl-producing bacteria and, secondly, supplies inorganic phosphate for subsequent use by the yeast population.

Some brewers using this wash appear to maintain a low diacetyl level in their product and attribute this to the phosphoric acid treatment.

METHODS

Two series of brews were fermented. One series was inoculated with a yeast population which had been washed with phosphoric acid each time prior to use; the other brew series was inoculated with an unwashed yeast population. Diacetyl determinations were made on each of the brews at the end of the fermentation cycle.

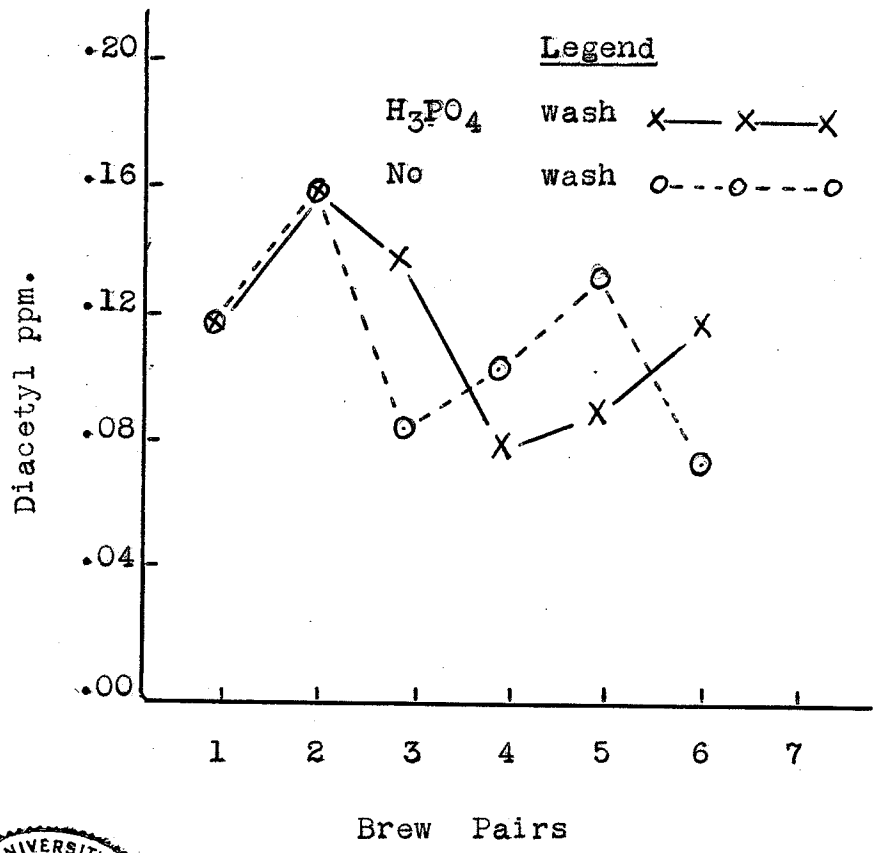
RESULTS AND DISCUSSION

Results of this part of the study are presented in Fig. 10. No significant difference in diacetyl levels appeared in the brews using a phosphoric acid wash as against those on which this treatment was not applied. If diacetyl levels were accentuated by bacterial contamination of the yeast inoculum, the use of phosphoric acid could assist in reducing the level of diacetyl to that which might normally be produced by the yeast population.

The increased level of inorganic phosphate

supposedly supplied by the phosphoric acid treatment seemed to have no significant effect upon diacetyl production although at times it appeared to stimulate the fermentation process.

Fig. 10. Diacetyl levels produced by phosphoric acid washed yeast and unwashed yeast.



SUMMARY

Certain factors which might influence the production of diacetyl in brewery worts were investigated and with the following results.

(1) In the absence of bacterial contaminants and respiratory deficient mutants diacetyl may be produced as a by-product of the normal fermentation cycle.

(2) Maximum levels are reached concomitant with maximum fermentation activity and when the bulk of the fermentable extract has been utilized.

(3) As the yeast enters the resting stage, diacetyl is removed by some unknown mechanism which might be related to a change in nitrogen metabolism.

(4) Accelerated fermentations where initial temperature was changed from 50° F (10° C) to 54° F (12.2° C) and holding temperatures from 56° F (13.3° C) to 60° F (15.6° C) had no noticeable affect upon diacetyl production.

(5) Successive fermentations with an initial and

holding temperature of 60° F (15.6° C) were accompanied by a rapid rise in diacetyl level after the third generation. The increase in diacetyl might be the result of a change in yeast metabolism brought about by the higher temperatures during the reproductive phase.

(6) Phosphoric acid washing of the yeast has no effect upon diacetyl production if the yeast in use is free from bacterial contaminants.

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