Effects of Fermentation Environment on Yeast Lipid Synthesis[1]

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ABSTRACT

The goal of the present investigation was to study the influence of various brewing parameters on the total lipid content as well as the fatty acid composition of the yeast. The effects of fermentation temperature, aerobic *vs.* anaerobic growth, yeast strain, and adjunct level were explored. The results revealed that yeast contained more lipids and more unsaturated fatty acids when grown at lower temperatures. Aerobic growth conditions increased the amount of unsaturated acids and sterols. The fatty acid composition of three yeast strains displayed some differences when grown under identical conditions and the total lipid content rose markedly when increasing portions of corn syrup were used for wort production. In an effort to determine the site of these lipid changes, yeast cells were homogenized and subjected to a differential centrifugation. Three different subcellular particles were obtained and analyzed for lipids. An attempt was made to correlate some of these findings with the accumulation of ethyl acetate in the resultant beer.

Key words: *Adjunct, Ethyl acetate, High-gravity brewing, Lipids, Yeast.*

Beers produced according to the concentrated wort procedure contain relatively high levels of acetate esters and are therefore difficult to match with existing normal gravity products (1). In our efforts to control ester formation in fermentations of high-gravity worts, we discovered a correlation between ethyl acetate in beer and yeast lipids. It was observed (18) that fermentations of worts containing high levels of fermentable carbohydrates and low levels of assimilable nitrogen resulted in beers with reduced ethyl acetate levels. After the corresponding yeast crop was harvested and analyzed, a high lipid content was noted.

Lipids are an integral part of cellular membranes and, consequently, changes in lipid composition are associated with changes in membrane characteristics such as fluidity and permeability (14,19). Membranes control the exit and entry of various metabolites within cell organelles and, by doing so, may participate in the first reaction of a multi-enzyme sequence. Also, penetration of an end product of a multi-enzyme sequence through intracellular membranes may cause a feedback inhibition and thus affect the overall biological processes.

Almost all beer flavor components are fermentation by-products and must pass through at least one membrane system, the plasma membrane. It is therefore not unreasonable to assume that the accumulation of these components might be controlled by the physiological state of such membrane systems. It therefore seemed appropriate for us to investigate the influence of various brewing parameters upon the lipid composition of the resulting yeasts. Specific factors such as fermentation temperature, aerobic *vs.* anaerobic growth, and type of yeast strain used were explored. The lipid-accumulating effect in the yeast, as a result of employing increased adjunct levels, was also studied. In this context, it was of particular interest to determine whether the lipids in question were localized in one or another of the major subcellular organelles such as nuclei, mitochondria, and microsomes. It is hoped that investigations of this nature may eventually provide an explanation for the correlation of acetate ester levels in beer and the lipid-accumulating effect observed in the yeast.

EXPERIMENTAL

Fermentations

Pilot brewery worts as well as plant worts were fermented with yeast normally used for commercial beer production. The fermentations were carried out isothermally at 20°C with *Saccharomyces cerevisiae* and at 14°C with *S. carlsbergensis* in 1.2-l. or 40-l. glass fermentors. Immediately after pitching (0.25 g/100 ml pressed yeast), the wort was purged with pure oxygen at a rate predetermined to raise the wort oxygen level to 8 mg/l.

Various wort adjunct levels were obtained by blending proportionate amounts of corn syrup (68% dextrose equivalents) with all malt worts.

The yeast was harvested as soon as the final attenuation was reached (stationary phase), and freeze-dried for safe storage.

Isolation of Cell Fractions

Harvested yeast cells were washed with cold distilled water, pressed, and suspended in 0.1 *M* tris-HCl buffer, pH 8.0, containing 0.1% bovine serum albumin; the suspension was then added to a homogenizing flask containing ballotini beads. The sample was shaken at maximum speed for 45 sec on a Braun Homogenizer and cooled with carbon dioxide. The disintegrated material was separated by differential centrifugation into mitochondria, nuclei, microsomes, and cytosol (6). A scheme of the differential centrifugation procedure is given in Fig. 1.

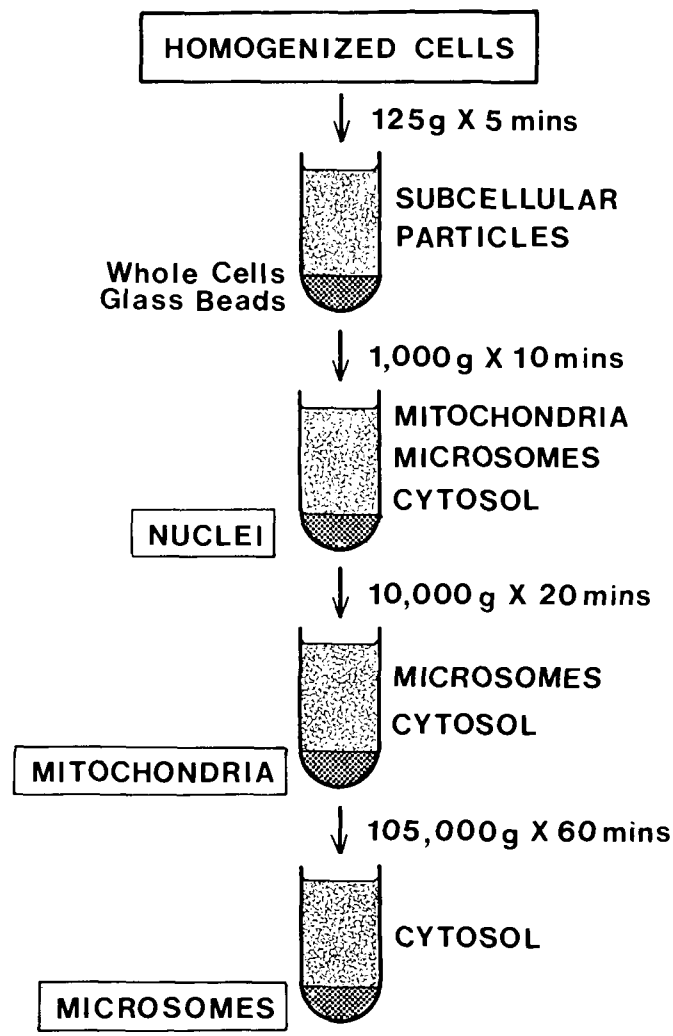

Fig. 1. Differential centrifugation.

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Isolation of Total Lipids from Cell Fractions

The cell fractions were extracted directly in the wet state by a modification of the method described by Kates (11). A 50-ml suspension of cell fraction, containing up to 750 mg dry weight in tris-HCl buffer was stirred at room temperature with 200 ml chloroform:methanol (3:1, v/v) for 16 hr on a magnetic stirring apparatus. The mixture was filtered and the cell material washed twice with chloroform:methanol. The filtrate and washings were combined and the organic phase was separated and brought to dryness in a tared round-bottom flask using a rotary evaporator. Residues were dried over KOH pellets in a vacuum desiccator.

Isolation of Total Lipids from Lyophilized Whole Yeast Cells

Extraction of whole freeze-dried yeast cells was carried out using a modification of the method described by Noble and Duitschaever (16). Aliquots of freeze-dried yeast (4 g) were ground to a fine powder, then suspended in 160 ml chloroform:methanol (3:1); the mixture was then stirred magnetically for 16 hr at room temperature. Total lipids were obtained by the work-up described above.

Saponification of Total Lipids

Total lipid extracts were treated in 80 ml 90% methanolic potassium hydroxide (33%) for 5 hr under reflux. The reaction mixtures were then saturated with sodium chloride to reduce the formation of gel, and 5 mg of n-nonadecanoic acid (used to determine extraction efficiency) was added. The nonsaponifiable lipids (including the sterols) were removed by extraction with three aliquots of petroleum ether (boiling range 30°–60° C). The reaction mixture was then acidified to pH 1.0 with HCl and the fatty acids extracted with petroleum ether. Both sterol and free fatty acid extracts were washed with deionized water, then filtered through anhydrous sodium sulfate. The extracts were reduced to dryness at 40°–50°C using a rotary evaporator. Small portions of benzene were used during evaporation to ensure that all water was removed. Residues were stored over KOH pellets in a vacuum desiccator.

Fatty Acid Analyses

The fatty acid residue was dissolved in chloroform and the solution made up to 50 ml. n-Heneiconsanoic acid (2 mg) was added as internal standard to 10 ml of the fatty acid solution, and the solvent was evaporated in a stream of nitrogen. The acids were methylated using 2 ml Methyl-8 reagent (Pierce Chemical Co.) and the esters separated by gas chromatography.

Sterol Analyses

The sterol analysis of the nonsaponifiable extract was by a modified procedure of Gordon *et al.* (5). Aliquots of the nonsaponifiable fraction containing the sterols and cholesterol (2 mg) as an internal standard were dried, redissolved in benzene, and analyzed by gas chromatography.

Gas Chromatography

Instruments: Perkin Elmer 990 gas chromatograph with flame ionization detector. Hewlett Packard Integrator Model 3380A.
Condition for fatty acids:

Column length:	6 ft, glass
Column o.d.:	6 mm
Column i.d.:	2 mm
Stationary phase:	10% SP-2340 (Supelco Inc., Bellefonte, Pa.)
Support:	Chromosorb W, AW
Mesh:	100/120
Carrier gas:	Helium
Inlet pressure:	80 psi
Column flow:	20 ml/min
Hydrogen pressure:	20 psi
Air pressure:	30 psi
Injector temperature:	250°C
Column temperature:	195°C Isothermal
Detector temperature:	250°C
Chart speed:	1 cm/min
Attenuation:	LOG.

The esters were identified by their retention times relative to methyl ester standards (8), and were verified by G. C.-Mass Spectrometer analysis.

Conditions for sterols:

Column length:	6 ft, glass
Column o.d.:	6 mm
Column i.d.:	4 mm
Stationary phase:	3.6% JXR Silicone (Applied Science Laboratories, Inc., State College, Pa.)
Support:	Gas Chrom Q
Mesh:	100/120
Carrier gas:	Helium
Inlet pressure:	80 psi
Column flow:	40 ml/min
Hydrogen pressure:	20 psi
Air pressure:	30 psi
Injector temperature:	280°C
Column temperature:	275°C
Detector temperature:	280°C
Chart speed:	0.5 cm/min
Attenuation:	LOG.

The sterols were identified by their retention times relative to those of sterol standards.

RESULTS AND DISCUSSION

Fermentation Temperature

One of the factors influencing fermentation performance is the fermentation temperature. The choice of temperature is partly

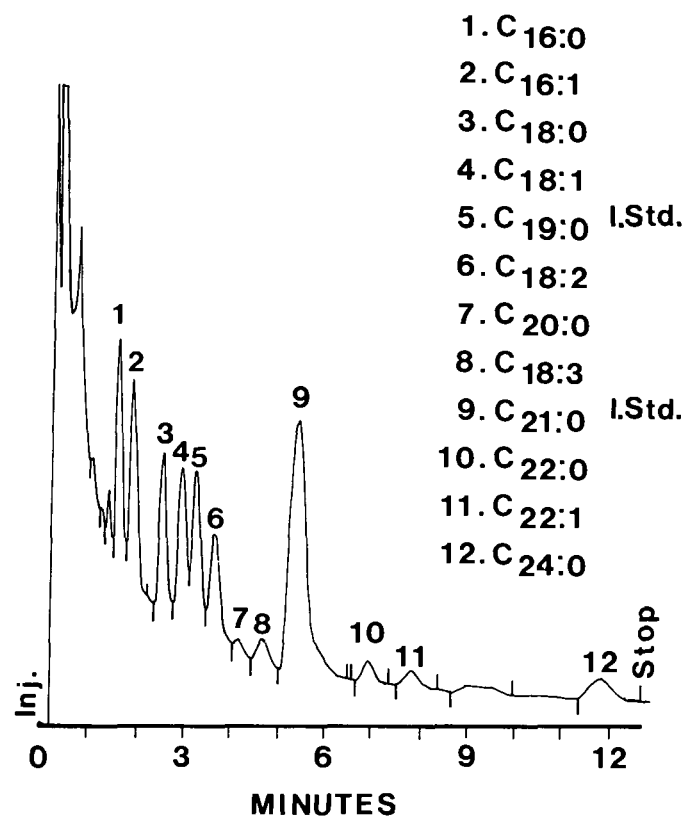

Fig. 2. Chromatogram of fatty acid methyl esters.

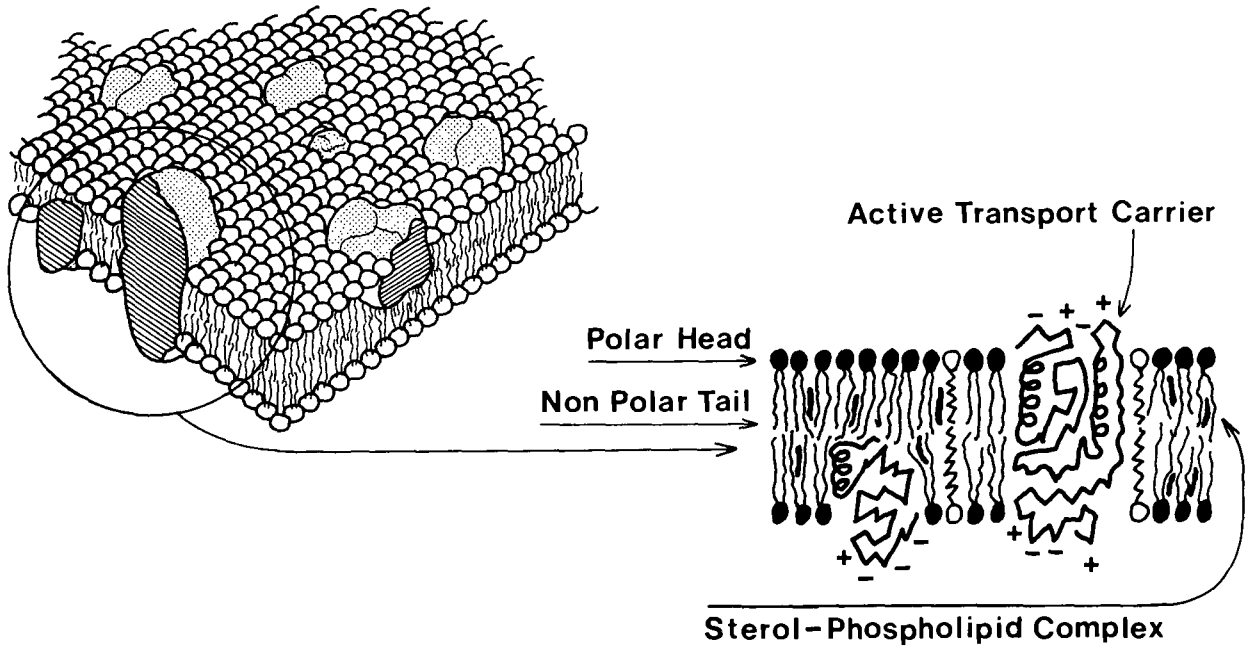

Fig. 3. Membrane fluid mosaic model (after Singer and Nicolson, ref. 22).

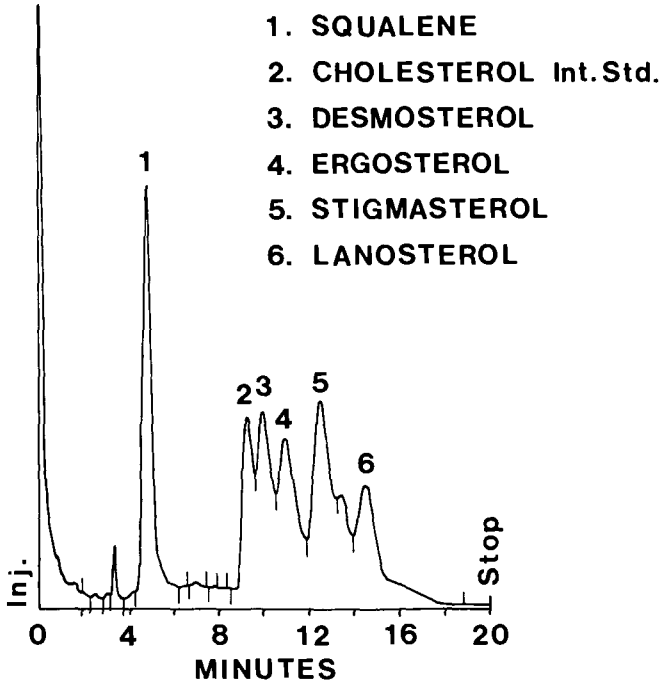

Fig. 4. Chromatogram of sterols.

dependent on the flavor and other desirable characteristics of the required beer, and usually lies within the range of 8°–20° C; for our investigations, three fermentation temperatures within this range were selected and their influence on total yeast lipids and fatty acids were studied. Figure 2 shows a typical chromatogram of the major fatty acids in yeast; Table I displays the effect of fermentation temperature on the proportion of saturated and unsaturated fatty acids. A distinct increase in total lipids was found whenever the temperature was lowered. Furthermore, it is notable that the increase in lipids occurred in the form of unsaturated fatty acids. Unsaturated fatty acids have a lower melting point than saturated fatty acids and it appears that there is a minimal requirement of unsaturated fatty acids for growth temperature. Similar

TABLE I
Lipids of Yeast Grown at Various Fermentation Temperatures (as per cent dry weight)

Fermentation Temp. °C	Total Lipids	Sat. Fatty Acids C_{16}, C_{18}	Unsat. Fatty Acids $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$
8	8.1	0.33	1.13
14	7.3	0.38	0.87
20	6.7	0.37	0.54

observations, although on *Escherichia coli,* were reported by Sinensky (21), who suggested that this compositional adaptation to environmental temperature serves to maintain constant membrane fluidity independent of the growth temperature.

In order to demonstrate the importance of membrane fluidity and its close relation to membrane transition, a model of a membrane is displayed in Fig. 3. This membrane model was proposed by Singer and Nicolson (22); the fluid mosaic structure of globular proteins within a lipid matrix appears to be the only model among those that have been suggested that is consistent with all the experimental data available so far. The filled circles represent the polar head groups of the phospholipid molecules, and the wavy lines represent the fatty acid chains; each molecule, protein as well as phospholipid, has a hydrophilic and a hydrophobic portion, a prerequisite for the basic membrane structure in the presence of water. Imbedded in the phospholipid bilayer are the catalytic proteins; this model explains the selective function of membranes and the means by which they regulate the passage of compounds into and out of the cell.

In their review, Singer and Nicolson (22) suggest that the nature of the fatty acids incorporated into phospholipids affects the function of certain membrane-bound proteins. In our experiments, we observed an increase in unsaturated fatty acids in yeast fermented at lower temperatures. This shift to a larger portion of unsaturated fatty acids may well be the result of a mechanism which produces membranes whose lipids maintain a constant fluidity independent of the growth temperature.

Aerobic *vs.* Anaerobic Growth

The role of oxygen in brewery fermentation needs no re-emphasis, as it has long been recognized that wort must be aerated in order to obtain a satisfactory fermentation. Hunter and Rose (7)

reported that *S. cerevisiae* requires oxygen to synthesize unsaturated fatty acids and sterols when grown under completely anaerobic conditions. Our investigations revealed similar findings when we grew *S. carlsbergensis* under agitation. Figure 4 shows a typical chromatogram of some of the sterols found in yeast as well as standards used for quantitative analysis, and Fig. 5 displays the effect of aerobic or anaerobic growth conditions on their composition. The most striking difference was the eightfold increase in unsaturated fatty acids when grown under aerobic conditions. The small amount of unsaturated fatty acid found in the anaerobic culture is probably vestigial traces of unsaturated fatty acids derived from the inoculum which was obtained from the brewery pitching yeast which had been previously exposed to oxygen. Characteristic of aerobically grown cells are the high level of sterols and the reduced level of squalene. The lower levels of sterols (especially of ergosterol) occurring under the anaerobic conditions reported here were accompanied by increased amounts of squalene; this finding has been reported by other workers (4,10,23). Sterols of course have been implicated in the physiological function of membrane systems and they appear to add stability to the membrane structure (20). Squalene may also influence the spacing of lipid molecules in membranes and hence influence membrane permeability characteristics (12). The oxygen that is available to yeast during fermentation merits careful consideration, particularly in view of the approach of Anderson *et al.* (2) to acetate ester control. In their studies, a reduction in acetate esters in beer was observed when highly oxygenated water was added during the latter part of fermentations.

TABLE II
Fatty Acid Composition of Three Yeast Cultures (in mg/100 g dry weight)

	Ale Yeast Top-Fermenting	Ale Yeast Bottom-Fermenting	Lager Yeast
C_{16}	300	320	330
$C_{16:1}$	800	920	700
C_{18}	120	115	80
$C_{18:1}$	240	370	120
$C_{18:2}$	43	45	115
$C_{18:3}$	5	6	15

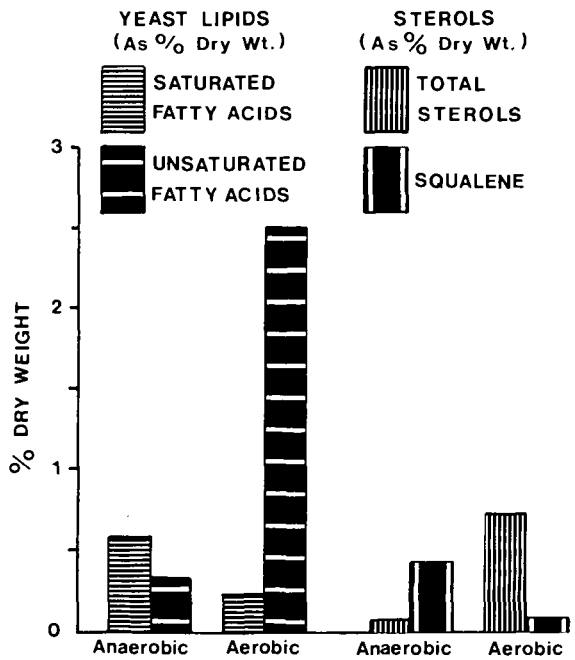

Fig. 5. Yeast lipids and sterols (as per cent dry weight).

Yeast Strain Differences

One of the brewing parameters that can be varied is the yeast strain. It was therefore of interest to us to determine the fatty acid profile of three yeast cultures which have been used for commercial beer production. In order to restrict the number of variables, all yeast strains were grown at the same temperature (14°C). Table II shows the fatty acid composition of these cultures. Generally, yeast shows a preponderance of C_{16} and C_{18} fatty acids, as we have verified with results shown here (19). Moreover, yeasts usually abound in monounsaturated fatty acids, especially 16- and 18-C atoms. The polyunsaturated fatty acids $C_{18:2}$ and $C_{18:3}$ are usually associated with specific yeast strains (9). Our data confirm this in that the ale yeasts (although one of them was bottom-fermenting) contain similar concentrations of linoleic and linolenic acids but the concentration of these acids is quite different in the case of the *S. carlsbergensis* strain. Finally, it is interesting to point out that it was primarily the level of unsaturated fatty acids which varied within these three yeast strains. The concentrations of the saturated fatty acids remained approximately the same.

Adjunct Level

Corn syrup is one of many raw materials which may be used to supplement malt in the grist bill. This adjunct material contributes only a small part toward the assimilable nitrogen content and an increased application of its use as adjunct actually dilutes the available nitrogen. Syrup used for brewing purposes usually contains high amounts of glucose (68 DE) and additions to the kettle inevitably change the overall carbohydrate spectrum of the resultant wort. In an earlier fermentation study (18), we noted that increased proportions of corn syrup in wort caused the total lipid content in the resultant yeast to rise (Fig. 6). In our efforts to pursue this matter further, we decided to determine just where in the yeast cell most of the lipids were being localized.

Freshly harvested yeast cells were homogenized and subjected to a differential centrifugation procedure which yielded pellets of nuclei, mitochondria, and microsomes. These major subcellular particles were analyzed for their fatty acid composition (Figs. 7 and 8). Both histograms display the amounts of fatty acids as a percentage of protein in the subcellular fractions for three different adjunct levels. Inspection of these data revealed a marked increase of unsaturated fatty acids in nuclei, mitochondria, and microsomes. However, the amount of saturated fatty acids rose in the nuclei but remained almost constant in the two other subcellular organelles. From these results, it becomes clear that the ratio of unsaturated fatty acids to saturated fatty acids varied according to the adjunct level only in the mitochondria and microsomes; both subcellular fractions are known to contain high

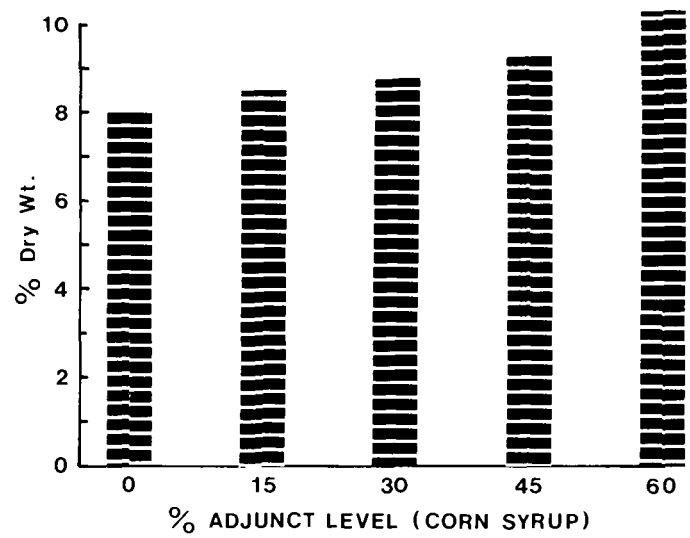

Fig. 6. Total lipids in yeast at various adjunct levels (as per cent dry weight).

amounts of membranous matter.

The importance of the ratio of unsaturated to saturated fatty acids is also emphasized in Table III. In this display, an attempt was made to correlate the increase in membrane fluidity (which is dependent upon the above-mentioned ratio) with the reduction of ethyl acetate in beer. Unfortunately, the present state of our investigations is not as yet far enough advanced to allow us to draw such conclusions. However, speculation along the following lines might be appropriate at this time.

According to Nordström (17), ethyl acetate is formed by a reaction of acetyl-CoA with ethanol and, as we have suggested in an earlier publication (18), any means which reduces this pool of acetyl-CoA in the cell may also reduce the concentration of ethyl acetate in beer. A possible utilization of acetyl-CoA molecules has been suggested by Mahler and Cordes (15) for mammalian cells grown in high glucose concentrations. Under these conditions (catabolite repression) certain enzymes of the TCA cycle are repressed and acetyl-CoA molecules are directed in large numbers toward lipid synthesis (Fig. 9). Catabolite repression is also observed in yeast cells when grown in media containing 5% glucose

TABLE III
The Ratio of Major Unsaturated to Saturated Fatty Acids in Brewer's Yeast and the Concomitant Concentrations of Ethyl Acetate in Beers of Various Adjunct Levels

% Adjunct level (corn syrup)	0	15	30	45	60
Ratio $\frac{\text{unsaturated}}{\text{saturated}}$	2.24	2.49	2.56	3.35	5.75
Ethyl acetate mg/l.	18	16	15.5	15	11

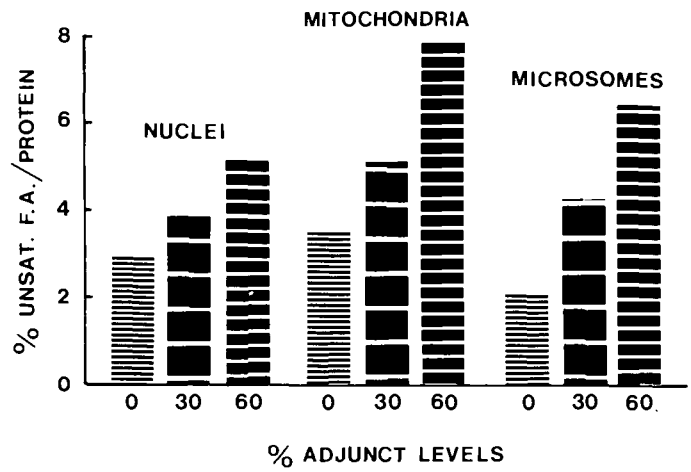

Fig. 7. Unsaturated fatty acids in subcellular particles (as per cent protein).

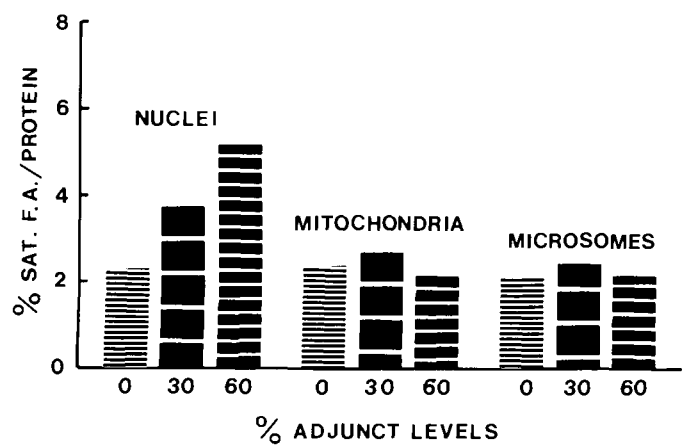

Fig. 8. Saturated fatty acids in subcellular particles (as per cent protein).

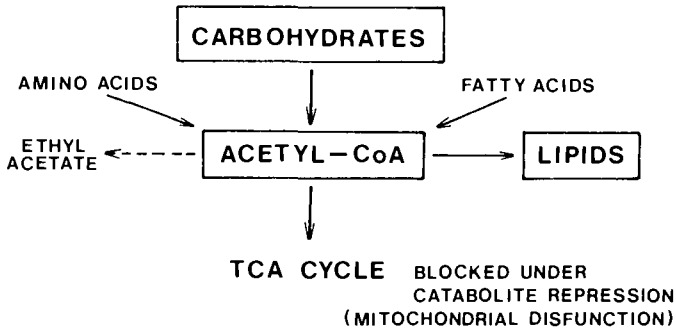

Fig. 9. Simplified scheme of lipid biosynthesis.

(3,13). These yeast cells showed fewer mitochondria than yeast grown in a 1% glucose medium and, in addition, the membranes of the mitochondria were difficult to resolve compared with other membranes of the cell (3).

Abnormal function of the TCA cycle might be expected to occur if the mitochondria were abnormal. If the TCA cycle were not functioning normally in catabolite-repressed yeast cells, one might expect more acetyl-CoA to be used for lipid synthesis. Indeed, in our fermentation experiments with worts of increasing glucose (*i.e.*, corn syrup) levels, we found that the resultant yeast contained more lipids (Fig. 6). In every case, the glucose concentration was certainly high enough to cause catabolite repression, and we propose that during such a condition acetyl-CoA molecules are directed more toward lipid synthesis than to ethyl acetate formation.

CONCLUSIONS

Brewing parameters such as fermentation temperature, aeration, yeast strain, and adjunct level have a marked influence on yeast lipids and their composition. In particular, the use of increasing proportions of corn syrup in wort resulted in yeast crops which contained more lipids as well as higher ratios of unsaturated to saturated fatty acids. Analyses of subcellular particles revealed a similar trend in mitochondria and microsomes, and concomitant with these was a reduction in ethyl acetate levels in the resultant beer. It is suggested that yeast cells under conditions of catabolite repression direct acetyl-CoA molecules more toward lipid synthesis than toward ethyl acetate formation.

Acknowledgments

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