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Conversion of cheese whey to yeast lipid and single cell protein

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by

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INTRODUCTION

The continuing increase in cheese production in this country has resulted in a corresponding and inevitable increase in whey production. In 1975, about 3.6 billion pounds of cheese were produced in the United States (81). About 10 lbs of whey are produced for every pound of cheese.

The previous practice of dumping whey in streams has been outlawed, and sewage treatment costs are prohibitive. Consequently, in the last five years there has been considerable research interest in finding economically attractive alternative uses for this valuable food product.

A major share of the nutrients in milk are left in the whey after cheese manufacture, and there has been considerable interest in incorporating whey in animal or human food. The composition of whey is 93.5% water, 4.9% lactose, 0.9% protein, 0.3% fat, 0.2% lactic acid, and 0.2% ash (86).

Raw whey can be fed in limited amounts to monogastric animals such as pigs or poultry (51). The high water content of whey may limit its use in feed since about three gal/day are required to supply the energy needs of a young pig. This unreasonably high fluid intake still does not meet protein requirements, so these animals require a high protein supplement in the ration. However, a substantial part of a ruminant animal's nutritional needs can be supplied by whey. Raw whey has been fed to dairy cattle that were slowly adapted to it (27). Increment feeding allows the microflora of the rumen to adapt to lactose and thus avoid bloating.

Whey is about 93% water and can be economically transported only short distances. Large cheese plants producing 50 to 100 million pounds

of whey yearly usually cannot find a cattle feeding operation nearby that will accept such large volumes of whey. Thus, further processing is necessary to remove the water. This can be done by drum or roller drying, or by membrane filtration or reverse osmosis.

Dried whey is routinely incorporated into animal or human food. This process is an economically marginal operation since the supply of dried whey vastly exceeds the demand. Nonetheless, most of the whey in the U.S. is dried and sold in this market (81).

The protein in the whey can be removed by membrane filtration. Whey proteins are by far the most usable component of whey, having a high protein value. The other components left after protein removal must be further processed in some way. The ultrafiltrate or permeate may be concentrated by reverse osmosis or dried and sold for incorporation into foods and feeds (81).

Modification of whey via fermentation has intrigued scientists for some time. The most popular processes have been the production of ethyl alcohol, lactic acid, lactase, and nonalcoholic and alcoholic beverages (85). However, most of these have had little consumer acceptance or commercial appeal in the U.S.

The fermentation of whey to lipid has been suggested, but not seriously considered, for about 60 years. This process is attractive for several reasons. Energetically, conversion of carbohydrate to fat is a balanced reaction and compared with general growth, requires few cofactors and minimal metabolic machinery. Most of the energy of the carbohydrate is conserved in the fat. The fat can be easily harvested and processed by

existing and readily available technologies. The residual cell mass could be treated as single cell protein and incorporated into feeds and foods.

The purpose of this research project was to develop a fermentation process for the production of lipid-rich microorganisms from whey and dairy plant wastes.

LITERATURE REVIEW

All living cells contain some lipid. Often the lipid is found entirely in membranes and organelles. Many organisms can also store energy as triglycerides. In higher plants and animals triglycerides have been exploited as a commercial source of food oils, e.g., olive oil, soybean oil and lard (88).

Microorganisms can also accumulate lipid as an energy reserve (87). Some bacteria accumulate poly β-hydroxybutyrate while most fungi and some algae accumulate triglycerides. These latter two groups have been suggested as an alternative source of food oil. Algae such as Chlorella sp. have been reported to accumulate up to 80% oil dry weight (73). But, algae have not been used in fermenting media such as whey that is rich in organic nutrients. Fungi have recently attracted attention as food oil producers from unusual substrates such as petroleum (67), but they have traditionally been grown on media rich in carbohydrates. There have been several attempts to produce fat from whey and a limited number of organisms have been studied for this purpose (66).

The earliest attempt to produce oil by microorganisms on a commercial scale was during World War I. This early process was done on broth in shallow trays or on straw or sawdust wastes on the floor (66) and has been referred to as the 'pan' or 'floor' process. Endomyces vernalis (now Trichosporon pullulans) yielded an oil like a typical vegetable oil when grown in this way.

Other organisms were used to produce oil from many substrates in the 1940's and 1950's and are discussed in a 1959 review by Woodbine (90).

The most popular organisms were Geotrichum candidum, Fusarium sp., Mucor circinelloides, Penicillium javanicum, Aspergillus ustus, T. pullulans, Lipomyces lipofer, Torula utilis and Rhodotorula glutinis. Of these, G. candidum (Oospora lactis) isolated from a sewage drain in the 1930's was probably the most successful fat-producer in whey. The whey medium was enriched with ammonium sulfate, potassium chloride, and magnesium sulfate, and after five days of cultivation yielded 14.34 g fat/100 g sugar (90).

The later work by Woodbine (90) on Penicillium and Aspergillus species grown on whey concentrated on reduction of biological oxygen demand (BOD), as well as fat production. He added ammonium nitrate to whey and found the organism (A. ustus) used 96% of the lactose and produced a pellicle yield of 17 g/L of whey which was 13% protein and 28% fat. The process took about 12 days.

The other organisms listed above used lactose poorly and were, therefore, not very successful in whey (31). Rhodotorula gracilis (now R. glutinis) was a popular organism for study because of its high fat content (∿60% dry weight) (26,45). Steinberg and Ordal (76) studied the production of fat by this organism from corn sugar in fermenters and their results are indicative of the most important features of fat formation by other organisms. They found that high aeration rates, pH and temperature control were important for rapid fat formation and high fat yields.

In 1974 Mickle et al. (57) used R. gracilis to lower the BOD of cheese whey and produce fat. Because R. gracilis is unable to assimilate lactose (50), they added 5% sucrose to the whey. Even though the added sugar was converted to fat, this was an inherently inefficient process for BOD

reduction.

Fat production by microorganisms growing on waste products other than whey has had some recent emphasis. Many different substrates have been used such as sweet potatoes (60), cane molasses (43), beet molasses (17), sulfite wastes (24), cellulose wastes and hydrolyzed wood (90) and Iraqi date extract (59).

Microorganisms

The genera that are most often reported to be fat producers are the yeasts Rhodotorula and Lipomyces and the molds Aspergillus and Penicillium. Many other organisms have been shown to produce fat as well in other media besides whey, and there are 'fat' species of certain genera. However, the capacity to produce large amounts of storage fat varies with the species and strain. Since fat production isn't a taxonomic criterion, these organisms are scattered throughout the genera. Table 1 lists some yeasts and molds that have been reported to produce at least 35% fat dry weight (87).

Culture Conditions

Physical conditions

The yields and composition of the fat produced in any process depends on the conditions of cultivation and the composition of the medium. The influence of physical conditions such as temperature or pH are primarily on the rate of metabolism while aeration can influence the efficiency of metabolism.

For example, the efficiency of fat production is greatly enhanced by adequate aeration. Many of the organisms used for fat production such as Rhodotorula sp. and Trichosporon sp. are obligate aerobes and thus depend

Table 1. Total lipid content of fungi[a].

Organism	Total lipid (% dry wt.)
Mucor albo-ater	6.5-41.8
M. circinelloides	15.7-45.4
M. spinosus	28.4-46.2
M. ramannianus	16.7-55.5
Rhizopus sp. (I)	11.6-45.3
Aspergillus fischeri	10.5-37.0
A. flavipes	39.7
A. flavus	5.7-35.5
A. nidulans	14.8-39.8
Chaetomium globosum	54.1
Fusarium lini Bolley (1)	5.9-34.6
Mortierella sp.	34.9
M. vinacea	3.2-51.4
Paecilomyces lilácinum	6.0-47.3
Penicillium javanicum	17.5-41.5
Trichosporon cutaneum	45.0-56.0
Ustilago zeae	30.2-36.6
Rhodotorula gracilis	20.3-63.2
Saccharomyces cerevisiae (ATCC 7754)	68.5-87.1
"Soil yeast"	5.5-65.3

[a]Representative data that have been collected and published by Weete (87).

on aeration for growth (50). Other yeasts capable of alcoholic fermentation in general produce more fat in highly aerated cultures. Aeration stimulates oxidative rather than fermentative metabolism (Pasteur effect) and influences an organism to produce fat rather than some partially oxidized end product.

The positive influence of aeration on fat production was appreciated early (90). The 'pan' and 'floor' process for growing T. pullulans took advantage of this principle. The modern fermenter capable of high aeration rates was an improvement over previous cultivation methods.

The stimulation of fat production by adequate aeration may be attributed to two factors. The most obvious influence is the positive effect on growth rate (47). Cells can grow rapidly to maximum cell numbers and then begin to deposit fat. High aeration rates during the fattening phase may be effective in removing CO_2 in a flushing action rather than by supplying oxygen. High concentrations of CO_2 may be the volatile inhibitory factor discussed by some authors (18). Low levels of CO_2 may actually stimulate fat production (13).

Most organisms produce maximum fat at or near the optimum pH for growth (87). An exception to this is R. gracilis which showed an optimum pH for fat production at 8.0. However, others reported an optimum pH of 5.5 to 6.0 for fattening (45).

Temperature can have a marked effect on the amount of fat produced and the fatty acid composition. Some authors have reported higher fat yields at temperatures above or below the optimum for growth, but most investigators report that the optimum temperature for fat production and

growth are similar. For example, the fat production of R. gracilis was cut in half by lowering the temperature from 28C, the optimum growth temperature, to 22C (76).

The fat of plants and animals is believed to be partially or wholly liquid at their growth temperature (21). As the growth temperature of an organism is lowered, most organisms alter the fatty acid composition being produced to maintain partial fluidity. The plasticity of fats and the phase changes of membrane lipids with temperature have been recent areas of interest and the subjects of several reviews (14,21,25,80). It has been shown, for example, that membrane lipids of Escherichia coli must maintain at least one-third of the normal fluidity or the cell will die (21). Thus, the ability of a cell to alter its fatty acid composition is crucial for survival.

Theoretically there are several ways a cell can control the melting point of its fat. The melting point of homologous fatty acids is directly dependent on chain length with shorter fatty acids having the lower melting point. Secondly, unsaturation of a fatty acid lowers its melting point, with cis double bonds being lower than trans, and polyunsaturated lower than monounsaturated (46). Bulky side groups such as branched fatty acids will also lower the melting point to about the same degree as a cis double bond. When the triglyceride is composed of a homogenous mixture of fatty acids, the melting point will be sharp. If a heterogenous mixture of fatty acids is present, the fat will have a broad melting point (84).

The changes in yeast fatty acid composition with temperature have been found to follow these general rules. Of the methods available to the

yeast to change its fatty acid composition, increasing the degree of unsaturation at lower temperatures is the one observed most frequently. For example, a decrease in growth temperature from 25 to 10C resulted in a higher ratio of linoleic acid (18:2) to oleic (18:1) for Candida lipolytica (42). Reports of lowering melting point by production of shorter chain fatty acids has been reported for Saccharomyces cerevisiae (38) which is unable to synthesize polyunsaturated fatty acids anaerobically.

Rising growth temperatures seem less dangerous to microorganisms than falling ones as far as fat composition is concerned. But at higher growth temperatures, the melting point of the fat being produced rises.

It has also been reported that psychrophilic, mesophilic and thermophilic fungi when grown at various temperatures alter the proportion of unsaturated fatty acids to adjust the fluidity of their fat (58). Some psychrotrophs, however, also produce some short chain fatty acids (87).

Nutrient conditions

The nutrient requirements for fat production are in general not much different from those for growth of the organism. Media deficient for adequate growth will in general be deficient for fat production. The effects of vitamin deletion on fat production are pronounced only if the organism requires that vitamin for growth. In general, in vitamin deficient media, an auxotrophic organism will produce drastically lowered levels of fat (69). One exception to this is an inositol requiring strain of Saccharomyces carlsbergensis which produces more fat in inositol deficient media than in inositol sufficient media (48).

Mineral additions have been shown to increase fat yields in some cases. Magnesium additions have stimulated fat production in media otherwise sufficient for growth (29). Phosphorus deficiency has stimulated fat production in some organisms (29) but decreased fat yields in others (90). It seems likely that adequate phosphorus for growth is adequate for fat production. Other minerals have been shown to have some positive influence on fat yields. However, their effect was not separated from their growth stimulating influence.

The nitrogen source in the medium may have an influence on the fat content of organisms. All yeasts can utilize ammonia salts, but only some can use nitrate (50). It has been shown that yeasts produce more fat from NH_4^+ than from NO_3^- (32). In some cases organic nitrogen may be superior to inorganic sources (87) but must be supplied as a protein hydrolysate as most yeasts are not very proteolytic (50).

The most important influence on the amount of fat produced is the ratio of carbon to nitrogen in the medium. If the C:N ratio is maintained, but the total carbon and nitrogen are increased, more cell mass will be produced but not more fat (15). However, if the carbon content is increased to a higher level while the nitrogen level is held constant, more fat will be produced. Fat-producing cells use up the available nitrogen to produce a cell mass and then produce fat from the remaining carbohydrate. For example, R. gracilis will produce up to 60% fat dry weight at very high C:N ratios (7). In studies of M. circinelloides (8) and Penicillium and Aspergillus species (61) the ratio of C:N can be controlled to produce fat-rich or protein-rich cells.

The carbohydrate used for an energy source in the culture medium can also influence the fat yield. There has been some interest in using hydrocarbons for fat production. The n-alkanes of chain length C_9 to C_{18} have been used by the fat-producing members of Candida and Torulopsis. The metabolism of these hydrocarbons and the fat produced are discussed in a recent review by Rattray et al. (69).

In general, glucose or maltose are efficiently used by fungi while lactose and pentoses are considered inferior (87) probably because of poor growth of many on these substrates (50). The level of glucose present in the medium is directly proportional to the amount of fat produced by the fat molds. For example, increasing the glucose concentration in the medium from 1 to 70% increased the fat produced from 10.4 to 36% dry weight for Aspergillus fischeri (67).

The level of carbohydrate present in the medium can have a profound effect on the metabolism and end products of some yeasts. In adequately aerated media, yeasts will use glucose oxidatively if the concentration does not exceed about 1.0% (47). When the concentration of glucose is raised, some yeast species will exhibit a repression of their oxidative metabolism and begin to use the glucose fermentatively. This form of repression in yeasts has been termed the Crabtree effect and those yeasts which demonstrate it are Crabtree positive (23). At high glucose concentrations, these yeasts produce ethanol, acetaldehyde and acetic acid and accumulate less fat than at low glucose concentrations.

Other yeasts can maintain a high rate of respiration at high glucose concentrations and are therefore Crabtree negative. Most of the Crabtree-

negative yeasts will not use the residual sugar in a growth medium but leave it unmetabolized; however, some of the yeasts can use the residual sugar to make fat (39).

The Crabtree effect is an interesting control for the yeasts; however, it is not an all or none phenomenon. The degree to which a Crabtree-positive organism switches to fermentative pathways depends on the species and the carbohydrate source. This change has been measured conveniently as a respiratory quotient or R.Q., which is the moles CO_2 produced divided by the moles O_2 used. When oxidative metabolism predominates, the R.Q. is 1 because:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O.$$

When alcoholic fermentation is present no oxygen is used and CO_2 and ethanol are produced. In a study of some species of yeasts growing on glucose, DeDeken (23) reported that the range of R.Q.'s was 4.1 to 100. Thus, some yeasts were only weakly Crabtree positive. Some strains of the weakly Crabtree-positive organisms studied by DeDeken (23) have been shown to be fat producers by others (87).

Although the work in this area is very limited, it is possible to deduce that fat-accumulating yeasts, i.e., fat content greater than about 20% dry weight, have these characteristics in common: they are either obligate aerobes, or if they are capable of fermentation, are Crabtree negative; they can use high concentrations of carbohydrate oxidatively; and, they can produce high concentrations of fat.

Yields of Lipids

There have been frequent reports of the cell and fat yields of R. gracilis grown on glucose. Enebo et al. (26) described a process in 1946 in which 4% invert sugar was fermented in 70 hr to yield 10 g/L cell mass which was 53% fat. The weight of fat produced per unit weight of sugar used x 100, or fat coefficient, was 18. Three years later Pan et al. (63) fermented a 4% glucose medium for 96 hr and produced 12.0 g/L cells which were 60% fat, and observed a similar fat coefficient of 16-18. Steinberg and Ordal (76) developed a faster process (43 hr) using this organism under controlled conditions of pH, temperature and aeration and produced 14.6 g/L cells dry weight which was 47.6% fat. In 1964 an in-depth study by Allen et al. (3) indicated that a fat coefficient of 40 was attainable but for only a short time. The most recent study (45) took only 60 hr to produce 10 g dry weight/L which was 66% fat. The fat coefficient was 17.1.

Other organisms have been reported to have similar efficiencies but in general do not reach such high levels of fat.

Lipids Produced

The lipid classes produced by fungi include aliphatic hydrocarbons, fatty acids, sterols, acylglycerides, glycosylglycerides, phosphoglycerides and sphingolipids (69). The total lipid content of most fungi ranges between 7 and 15% with only the so-called fat yeasts and fungi reaching 30-60%. Each class of lipids seems to have a specific function in the cell. The spores of fungi are often coated with a waxy hydrophobic coating (87). The interior of the spore may contain a large acylglyceride storage

vacuole. The phospholipid fraction functions in the transport processes and metabolism of the cell (21). However, the main lipid class which changes in relative abundance in fat yeasts is the triglyceride fraction, reaching up to 80% of the total lipid (39).

The fatty acid composition of triglycerides in yeast is similar to those found in other biological systems. They consist of a homologous series of saturated and unsaturated aliphatic acids, ranging in chain lengths from C_{10} to C_{24} with C_{16} and C_{18} the most common (37). Palmitic is the predominant C_{16} acid and oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) are the major C_{18} acids. Unusual fatty acids such as hydroxy, branched and highly unsaturated fatty acids occur but are relatively rare (41).

Different groups of fungi differ from each other in their fatty acid composition (87). For example, the Acrasiales or cellular slime molds contain a high amount of mono- and diunsaturated fatty acids that are conjugated rather than the methylene interrupted series normally found. The Phycomycetes produce a significant proportion of their lipids as γ-linolenic acid ($C_{18:3}\Delta^{6,9,12}$) rather than α-linolenic acid ($C_{18:3}\Delta^{9,12,15}$). In addition to this anomoly, the different classes such as the higher Zygomycetes and lower Oomycetes differ in their degree of unsaturation, but all seem to produce more unsaturated fatty acids than other fungi (87).

The Ascomycetes generally do not accumulate long chain polyenoic fatty acids, but rather accumulate mono- or dienoic acids with $C_{18:1}$ and $C_{18:2}$ the most predominant and relatively low concentrations of $C_{18:3}$ (41). The Basidiomycetes do not produce characteristic fats but tend to resemble the Ascomycetes (87).

Fatty Acid Metabolism

An excellent review of fatty acid synthesis and degradation is given by Weete (87). Only the most important points will be summarized here.

Fatty acid synthesis has been studied in detail by several scientists, the most famous of which is the Nobel Laureate of 1964, Feoder Lynen. His studies (53) of yeast fatty acid synthetase indicated that the overall catalyzing reactions are:

1. $\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \longrightarrow \text{Malonyl-CoA} + \text{ADP} + \text{Pi}.$

2. $\text{Acetyl-CoA} + n\text{Malonyl-CoA} + 2n\text{NADPH} + 2nH^+ \longrightarrow$

   $CH_3(CH_2\text{-}CH_2)_n - CO - \text{CoA} + nCO_2 + n\text{CoA} + 2n\text{NADP} + nH_2O.$

The addition of one C_2 unit involves eight different enzymatic steps. These enzymes are interesting in that they are bound together physically and the substrate is bound covalently to a carrier protein. The exact mechanism is still not fully explained, but it is believed that the enzyme complex is shaped somewhat like a wheel with the partially synthesized fatty acid extended from the hub by a carrier protein. This is free to rotate and pass the rim of the wheel where the enzymes are located. A single pass around the rim generates one C_2 addition onto the growing molecule. When the fatty acid chain length reaches 16 or 18 carbon atoms the fatty acid is released from the complex as a CoA ester. The exact mechanism of this release isn't well-known but may be due to stearic hindrance. The synthesis of longer chain fatty acids is apparently performed by a different complex (30). This biosynthetic sequence yields only saturated fatty acids. Unsaturation of fatty acids is an aerobic

reaction requiring NADPH, cytochromes, and a specific enzyme (69). The exact mechanism of this reaction isn't known but appears to be highly specific for introducing a double bond in the 9,10 position. There is an anaerobic pathway for unsaturation in bacteria but not in yeasts (87).

Polyunsaturated fatty acids in plants and yeasts are synthesized by terminal methyl directed desaturation producing $C_{18:2}(\Delta^{9,12})$. Animals can't desaturate fatty acids and hence require them in their diet.

There appear to be two main sites for the control of fat synthesis: acetyl CoA carboxylase and fatty acid synthetase which are the enzyme systems catalyzing reactions 1 and 2.

Acetyl CoA carboxylase is controlled by allosteric regulation in S. cerevisiae. This enzyme is activated by citrate and Mg^{++} and inhibited by long chain acetyl-CoA thioesters (68).

The regulation of acetyl CoA carboxylase depends on the regulation of a second enzyme, isocitric acid dehydrogenase, which catalyzes the formation of α-ketoglutaric acid from isocitric acid. Apparently, this enzyme is subject to allosteric regulation by ATP and AMP. When the ATP level is high, the enzyme is inhibited and citrate accumulates. The citrate stimulates the acetyl CoA carboxylase system and more malonyl CoA is synthesized (33).

The regulation of the second enzyme system, fatty acid synthetase, is less well-understood. It is believed to be stimulated by hexose phosphate (82) and inhibited by long chain fatty acids in animal tissues (82) and also to some extent in yeasts (69).

The control of the generation of excess NADPH (needed for reaction 2)

via the stimulation or inhibition of the pentose pathway has been studied in animal tissues. The control mechanism seems to involve the inhibition by citrate of phosphofructokinase of the glycolysis cycle. The glucose metabolized would then presumably be shunted through the pentose pathway and generate NADPH. This control mechanism has not been shown to exist in yeasts. In studies of Candida 107 (89) and R. gracilis (36), phosphofructokinase was absent during the growth and fattening phase. The authors concluded that the pentose pathway is the predominant metabolic pathway for these fat-producing yeasts.

Protein Production

After the fat is extracted from the cell mass, a residue remains which is high in carbohydrate and protein. Similar microorganism-produced protein or single cell protein (SCP) has been widely suggested as one solution to the world food shortage problem and is the subject of several reviews (44,49,73).

Production of SCP and its contribution to world food reserves has been a popular area of interest for several reasons summarized by Kihlberg (44). Microorganisms have a very short generation time and thus can provide a rapid mass increase compared with more conventional sources of protein. The protein content of microorganisms is high. Most contain between 7 and 12% nitrogen on a dry weight basis. After correction for nitrogen in purines and pyrimidines, it is still higher than most common foodstuffs. The production of SCP can be based on raw materials which are available in large quantities. The production of SCP can be carried out in continuous culture, independent of climatic conditions and with only a small land

area and water requirement.

The yield of cells and SCP depends on the substrate composition. For example, S. cerevisiae produced 23.2 g cells dry weight/100 g cane molasses; S. fragilis produced 55.0 g dry weight/100 g lactose while C. lipolytica produced 90 g dry weight/100 g mixed C_{14}-C_{17} n-alkanes (49). In most cases the nitrogen content of these media was insufficient and NH_4SO_4 was added to achieve optimum cell growth and protein production. The optimum C:N ratio for most organisms is similar. For example, it is reported to be 10:1 for S. fragilis grown in whey and 9.7:1 for C. tropicalis grown in hexadecane (49). The yield of protein under optimum conditions is about 54% dry weight for S. fragilis on cheese whey, and 66% for C. lipolytica on n-alkanes. These two organisms are only about 1.0 and 8.0% fat with most of the remaining dry weight carbohydrate.

The protein yield in yeasts which have a high percentage of fat is lower than in protein-rich yeasts. For example, Allen et al. (3) reported R. gracilis had a composition of 40% fat, 8.9% ash, 19.7% protein and 31.2% carbohydrate. If the fat were removed the protein would increase to 33% of the dry weight.

The nutritional quality of yeasts for human and animal consumption has been widely reported. The amino acid composition of the protein will determine its nutritional quality. Humans require lysine, leucine, isoleucine, valine, phenylalanine, tryptophan, tyrosine and methionine in their diets. Egg protein has been viewed by many to be the near 'perfect' protein in amino acid content and is often used as a standard protein. Yeast protein has been reported to have a good amino acid profile compared

to egg protein, but in general, is low in methionine and cysteine (16). This tendency was shown to be true for S. fragilis when grown in whey (49). Attempts to modify the amino acid composition or content of microbial proteins have had marginal success. There is no known way of increasing the protein content above the level of an actively growing cell. Since the proteins in a cell are directly involved with the active metabolism of the cell, drastic amino acid profile changes would tend to cause loss in viability (73).

Feeding trials with yeast indicate that it would best serve as a protein supplement. Best nutritional value would be realized when combined with wheat flour, corn, cottonseed or sesame seed flour (10).

Toxicity of some single cell protein (SCP) for animals has been reported to be due to deficiencies of selenium-containing factors and α-tocopherol. This has been reported to cause liver necrosis in rats and can be alleviated by adding α-tocopherol or selenium compounds (73).

The relatively high content of purine and pyrimidine (8-25 g NA/100 g protein) in SCP has caused some concern. The final metabolic product formed in man, primates and birds from the purine moiety of nucleic acids is uric acid. At physiological pH, uric acid is only slightly soluble and tends to precipitate out causing gout or arthritis. Excessive uric acid in the urine may be deposited as stones in the urinary tract. Calloway (12) concluded that the maximum safe intake for humans is 2 g nucleic acid/day corresponding to about 20 g/day SCP. Most mammals degrade uric acid to allantoin which is more soluble and easily excreted so the levels of nucleic acids aren't a concern in animal feed.

The nucleic acids can be removed from the SCP (either as whole cells or lysed cells) to prepare food-grade concentrates and isolates. Enzyme hydrolysis of nucleic acid by endogenous or added ribonucleases is used commercially for preparing Baker's yeast protein (71). The protein concentrates and isolates can then be prepared by precipitation of the proteins from disrupted cells. These isolates can be extruded into an acid solution (pH 4.5) to give protein fibers that can be useful in textured protein products (2). The functional properties of these proteins such as water and fat binding, emulsion stability, gel formation, and whipability have been reported to be good in some cases (83) and can probably be changed by chemical treatments such as succinylation (54).

There are still serious questions to be answered about the nutritional quality of SCP. For instance, little is known about tolerance or toxicity of other cell components such as cell walls, pigments, unusual carbohydrates, or steroids that are all likely to be ingested along with the protein (44). The effect of different kinds of processing on the digestibility and overall food quality are not known either. Finally, there is the problem of consumer acceptability. Nonetheless, in the last five years there has been considerable interest in producing SCP and several companies are now marketing this product (49).

MATERIALS AND METHODS

Fermenter Description

Fermentation studies were performed in a 14-L commercial fermenter (MicroFerm, New Brunswick Scientific Co., New Brunswick, N.J.). Aeration was achieved by pumping filtered air into the base of the fermenter (see Figure 1). Air flow rates were determined by an in-line rotameter. The pH was monitored with a Radiometer combined electrode (Radiometer, Copenhagen, Denmark) and controlled with a Radiometer automatic titrater (Type TITIC). The titrating solution was concentrated HCl. Occasionally when increases in pH were desired, 50% (w/v) NaOH was added through the inoculation port. The agitation rate was varied from 100 to 500 rpm. The temperature was controlled by a thermostat which circulated either hot or cold water through a coil. Dissolved oxygen was measured with a steam-sterilizable probe constructed according to Johnson et al. (40). This probe has a self-generated potential (silver-lead) and the change in potential with oxygen concentration was followed with a potentiometer (D. O. Analyzer, Fermentation Design, Allentown, Pa.) and a 10-mv strip-chart recorder (Houston Instruments, Bellaire, Tex.). Antifoam (Medical Antifoam AF Emulsion, Dow Corning, Midland, Mich.) was added, when needed, through the inoculation port.

To sterilize the fermenter jar about 2 g antifoam and 50 ml water were added and the jar was autoclaved for one hr at 121C. Nine liters of whole whey or whey permeate were autoclaved separately for 20 min. The pH and dissolved oxygen probes were autoclaved for 30 min.

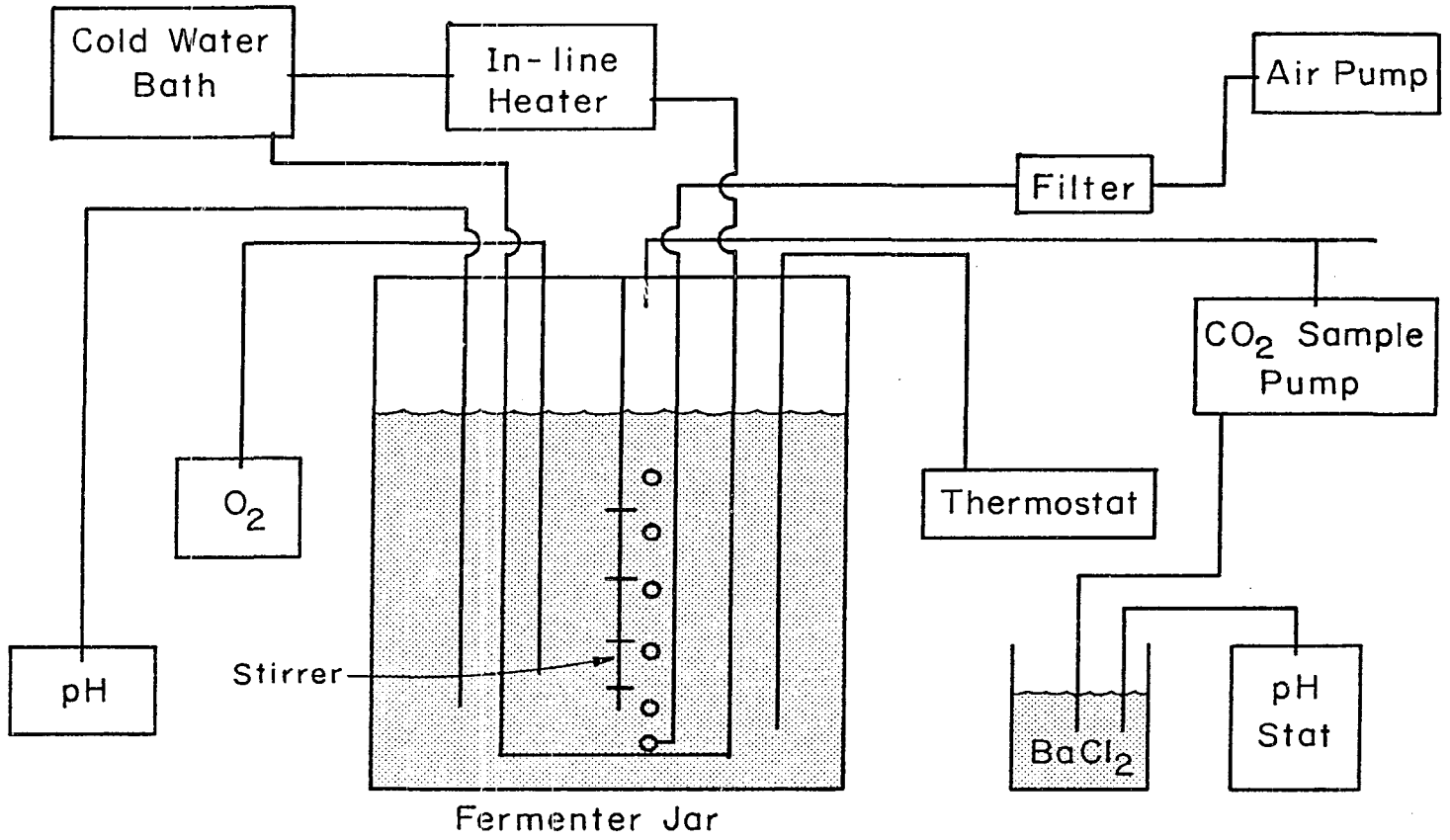


Figure 1. Schematic diagram of fermenter.

Immediately before beginning a fermentation, the medium was poured into the fermenter and the probes inserted. The medium was aerated for about 30 min to saturate it with air before a 10% inoculum of cells was aseptically added.

The fermenter inoculum was prepared by transferring one loop from a 24-hr malt extract slant to each of three 333-ml portions of fermentation medium in baffled 1-L Erlenmeyer flasks. The flasks were incubated at room temperature for 24 hr on a New Brunswick gyrotary shaker.

Carbon Dioxide Measurements

Exit gas from the fermenter was sampled (see Figure 1) using a variable speed peristaltic pump (Masterflex, Cole Parmer, Chicago, Ill.). The gas was pumped through a fritted disk gas dispersion tube into 75 ml of 0.50 N $BaCl_2$. The solution was maintained at pH 10.0 by an automatic titrater (Beckman Instruments, Fullerton, Ca.) adding 0.4 N NaOH. The $BaCl_2$ was changed after every 50-ml addition of NaOH to maintain an excess of Ba^{++}.

The determining reaction sequences are:

$$CO_2 + BaCl_2 \longrightarrow BaCO_3 + 2HCl.$$

$$2HCl + 2NaOH \longrightarrow NaCl + H_2O.$$

The mM CO_2/hr/10 L produced was calculated as follows:

$$\text{mM } CO_2/\text{hr}/10\text{ L} = \frac{(\text{N NaOH})(\text{ml NaOH/hr})(\text{ferm. air rate})RF}{2(\text{Sampling rate})}$$

where

Ferm. air rate = aeration rate in the fermenter in L/min.

Sampling rate = rate of flow of gas through the $BaCl_2$ in L/min.

RF = the recovery factor which is 1/fraction CO_2 recovered as determined in calibration experiments.

The accuracy of this method was tested by preparing gas mixtures with known amounts of CO_2 in the concentration ranges likely to be produced in the fermentation runs. The recovery of CO_2 varied from 37 to 77%, depending on the flow rates and CO_2 concentration in the exit gas. The slower the flow rate of the gas through the $BaCl_2$, the greater the % recovery of CO_2, and more dilute CO_2 concentrations were more efficiently recovered. The most efficient recovery (77%) was achieved at aeration rates through the fermenter of 10 L/min, and an exit gas sampling rate of 0.120 L/min.

This method of measuring CO_2 was superior to a gas absorption method developed and recommended by Phillips (65) for measuring R.Q.'s in aerobic cultures.

Oxygen Consumption

Dissolved oxygen measurements were used to measure the oxygen consumption of the microorganisms during the fermentation. When the air supply to the fermenter was stopped, the dissolved oxygen concentration fell linearly with time. The slope of a plot of oxygen concentration versus time represented the oxygen consumption rate of the microorganisms. By assuming the concentration of O_2 at saturation in the fermenter to be equal to that of O_2 in air-saturated water at 25C (5.78 cc/1000 cc), O_2 consumption rates were calculated.

Analytical Procedures

A 200-ml sample of fermentation broth was withdrawn from the fermenter at various times during the fermentation. Analyses, as outlined in Figure 2, were performed on these samples to follow the course of the fermentation. All analyses were not performed on every sample, but a suitable schedule of determinations was used to follow changes in the fermentation.

In the whey studies, dry weight, Kjeldahl nitrogen and amino acids were not determined because of the interference of precipitated whey proteins which contaminated the cells recovered by centrifugation. The fat/g wet weight of cells and the fatty acid composition were measured. The cell wet weight per ml was inadvertently not determined. To calculate fat production/volume of medium, a weighed sample of cell paste was resuspended in 100 ml distilled water and the cells/ml counted. This count was compared with the cell count during the fermentation to calculate the volume represented by a certain wet weight of cells.

Cell counts

A Max Levy mold-counting chamber was used to make direct microscopic cell counts. The slide contains etched squares 1/4000 mm^3 deep. Counts were made on diluted medium (usually 1:10 in distilled water). Three counts were made on each aliquot and these values averaged. The number of organisms/square was multiplied by the dilution factor (2.5×10^7) to obtain the number of organisms/ml. Cells which budded and produced pseudomycelium were counted as two cells when the daughter cell reached >1/2 the size of the mother cell.

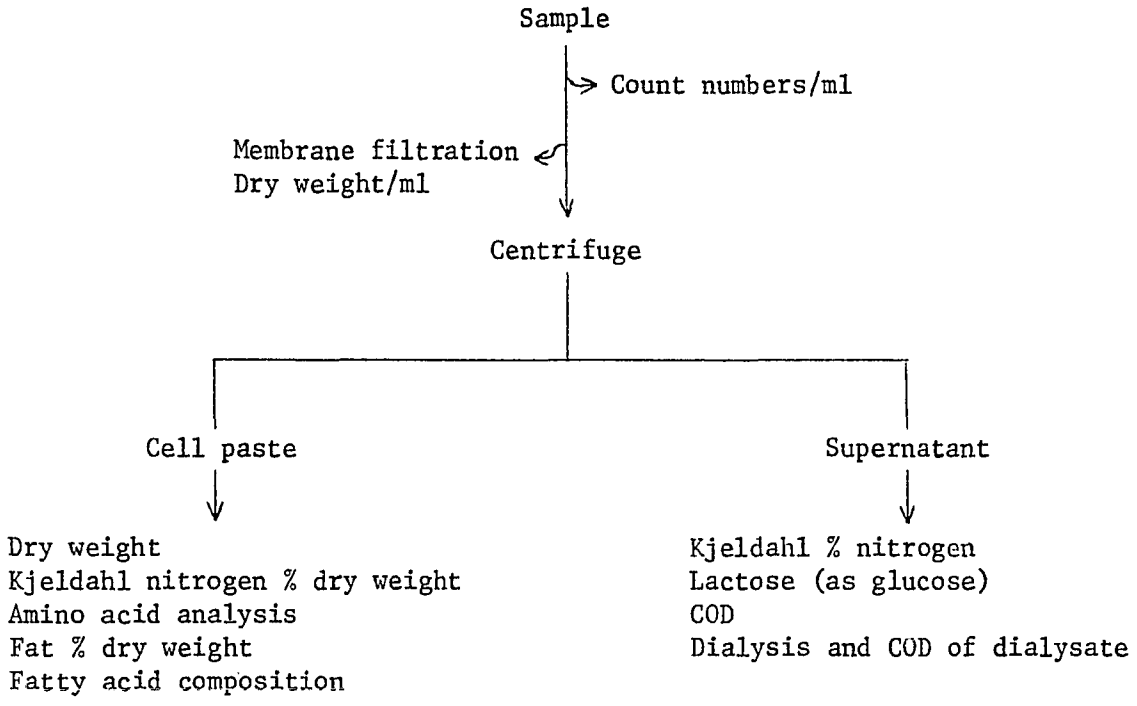


Figure 2. Schematic representation of fermentation broth analysis.

In early fermentation trials, counts were obtained by appropriate dilution and plating on potato dextrose agar. Direct microscopic counting gave a more accurate indication of cell growth because of the budding and mycelial growth pattern of all of these organisms.

Centrifugation

The cells were collected from the medium by centrifuging for 30 min at 7,900 x g at 5C in a Sorval RC2-B refrigerated centrifuge (Ivan Sorval Inc., Newtown, Conn.). Cells were washed once in distilled water, re-centrifuged and then frozen until analysis.

Dry weight of cells

To determine the dry weight of yeast/ml permeate fermentation medium, a 2 to 10-ml sample was filtered through a tared 0.45 μ membrane filter (Millipore Corporation, Bedford, Mass.). The filter and cells were washed once with 5 ml distilled water, dried in a vacuum oven for 12 hr at 45C and weighed. Uninoculated fermentation medium was also filtered and dried and used as a weight blank.

The dry weight of the cell paste was determined by placing cell paste in a tared aluminum pan and drying in a vacuum oven (12 hr at 45C).

Kjeldahl nitrogen

The Kjeldahl nitrogen content of whey, whey permeate and whole cells was determined by the method described by Bird (9).

Chemical oxygen demand (COD)

The chemical oxygen demand of centrifuged culture medium (7,900 x g, 30 min) was determined as prescribed by Standard Methods for the Examination of Water and Waste Water (4).

Dialysis of culture medium

A 10-ml aliquot of centrifuged (7,900 x g for 30 min) culture medium was transferred into prewashed dialysis tubing (Union Carbide Corporation, Chicago, Ill.) and the ends were secured. The sample was dialyzed against two changes of distilled water with a ratio of sample to dialysate of 1:500. After 18 hr dialysis at 5C, the samples were transferred to a 25-ml graduated cylinder and brought up to volume. Twenty ml of a 3:100 dilution were used for COD analysis.

Sugar determination

The glucose, galactose and lactose concentration of the spent fermentation medium was determined using the enzymatic method of Hettinga et al. (35). This method determines lactose as increase in glucose or galactose after acid hydrolysis of the sample. Only glucose determinations were made on most samples as they proved to be the most reliable of the two procedures and there was little free galactose.

Amino acid analysis

A modification of the method of Stewart (78) was used for amino acid analysis. A weighed sample of cells ($\sim$1.0 g) was placed in a tared lyophilization vial. Four ml of 6 N HCl were added and the tube was flame-sealed and autoclaved at 105C for 24 hr. The tubes were then cooled,

the contents quantitatively transferred (total wash volume of 50 ml distilled water) to a 125-ml separatory funnel and extracted with 20 ml petroleum ether to remove the hydrolyzed fat. The water layer was passed through a column of 5 g Dowex 50-X8 cation exchange resin at 5 ml/min flow rate. The column was washed with water until the pH of the effluent was 6.0. The cation exchange column was then eluted with 5 ml 6 M NH_4OH and then washed again until the pH of the effluent was 6.0. The sample and washings from the cation resin were then passed through a column containing 5 g of Amberlite IRA-400 anion exchange resin (OH^- form) at a flow rate of 5 ml/min. The anion exchange column was eluted with 5 ml 6% $(NH_4)_2CO_3$ and washed with distilled water until the pH again reached 6.0. The NH_4OH and $(NH_4)_2CO_3$ eluates were dried on a rotary vacuum evaporator and weighed. The amino acid composition was analyzed by the Department of Biochemistry and Biophysics, Iowa State University, using a Beckman amino acid analyzer (Beckman Instruments, Fullerton, Calif.)

Fat extraction

Several methods for the extraction of lipids from wet yeast cells were compared for their reproducibility, convenience and recovery. A 0.5-g sample containing at least 50 mg fat was used for all analyses.

Acid hydrolysis

The cells were hydrolyzed in 3 ml of 2 N HCl in a 70C wax bath for five hr and the digest was quantitatively transferred to a 200-ml glass centrifuge bottle. Then 30 ml chloroform-methanol (2:1) was added and stirred with a magnetic stirring bar for 30 min. After centrifuging the

supernatant solution was decanted into a 125-ml separatory funnel. The extraction procedure was repeated once and the solvent extracts pooled. The pH was adjusted to 7.0 with 2 N NaOH and distilled water added at 1/5 the volume of the solvent. The layers were allowed to separate. The chloroform layer was transferred to a tared vial and evaporated to dryness under a stream of N_2 to avoid fatty acid oxidation. The weight of the oil was recorded and calculated as % oil/g dry weight of cells.

Sobus and Holmlund method (74)

This benzene:methanol (1:1) extraction procedure was tested. The method was also modified to employ ethanol and hexane as an economical substitute solvent system.

Aqueous alkaline hydrolysis

Cells were hydrolyzed in 5 ml alcoholic KOH (12% v/v, 12% w/v respectively, in water) at 70C for two hr in a test tube sealed with a Teflon lined screw cap. The tubes were cooled and the pH adjusted to 2-3 by adding 1 ml concentrated HCl. The fatty acids were extracted with 5 ml hexane by mixing for 2.5 min with a vortex test tube mixer. A second 1 min extraction with 2 ml hexane was performed and the extracts pooled. The hexane was removed by evaporation under a stream of N_2 and the fatty acids weighed.

Method of Folch et al. (28)

This classic chloroform-methanol (2:1 v/v) extraction was used as originally described and also expanded to include different methods of physical disruption of the yeast cells during extraction. Cells were

sonicated (Blackstone Instruments, Sheffield, Pa.) at maximum intensity in the chloroform-methanol (2:1) mixture for 0.5 hr. The cells and extraction mixture were kept in an ice bath to avoid heating during sonication. One minute sonication periods were alternated with 30 sec cooling periods.

Cells were also disrupted in a Mickle disintegrator (Brinkman Instruments, Great Neck, N.Y.). The yeast was mixed with 0.5 g ground glass and the chloroform-methanol mixture for 0.5 hr. Heating was again minimized by placing the entire apparatus in a 4C walk-in refrigerator.

After disruption, the cells and debris were quantitatively transferred to a glass centrifuge bottle and centrifuged. The supernatant was transferred to a 125-ml separatory funnel. The cell pellet was reextracted twice by mixing for 30 min with the chloroform-methanol mixture using a total volume of chloroform-methanol 20x the total volume of sample plus transfer washings. Distilled water equal to 1/5 the volume of chloroform-methanol was added to the solvent mixture. The layers were allowed to separate and the lower chloroform layer removed to a tared vial. The solvent was again evaporated under a stream of N_2 and the fat weighed. The % fat/g dry weight of cells was calculated.

Fatty acid analysis

The fatty acids were methylated following the procedure of Luddy et al. (52). About 5 mg fatty acids were placed in a 10-ml screw cap vial and 0.3 ml of 14% (w/v) BF_3 in methanol was added. The vial was flushed with N_2 and heated in a water bath at 65C for five min. The vial was cooled and 0.5 ml 5% Na_2CO_3, 3 ml of water and 1 ml of hexane were added

to the vial and shaken. The hexane layer containing the methylated fatty acids was withdrawn and analyzed for fatty acid content.

Gas chromatography

Methyl esters were analyzed by gas liquid chromatography using a Beckman GC-5 chromatograph. The gas chromatograph was equipped with a flame ionization detector and on-column injection. The 6 ft x 1/8 in o.d. stainless steel column was packed with 15% EGSSX on 100/120 mesh Chromosorb P (Applied Science Lab, Inc., State College, Pa.). The operating conditions were: oven temperature 185C; gas flow rates of helium, hydrogen and air 50, 40 and 300 ml/min, respectively. The samples were compared with a standard fatty acid mixture containing 10% palmitic, 5% stearic, 30% oleic, 50% linoleic and 5% linolenic prepared by Nu Check Prep. Inc. (Elysian, Mn.).

Culture Maintenance

All cultures were maintained on malt extract agar. They were routinely transferred once a month to a fresh slant, incubated at 25C for one week, and then stored at 4C.

Fermentation Media

Dried whey (Formula 521, Associated Milk Producers) was reconstituted to 6.5% solids. It was frozen until needed for fermentation trials.

The whey permeate was supplied by the Mississippi Valley Milk Producers (Luana, Ia.). It was prepared with a Dorr-Oliver (Stamford, Conn.) ultrafiltration unit equipped with polysulfone membranes having a 24,000 molecular weight rejection limit. The permeate was concentrated about 3:1

to increase ease of handling. It was frozen until needed for fermentation trials. As needed, it was thawed, diluted and autoclaved for growth studies.

Culture Isolation and Selection

Yeasts and molds suitable for fermenting whey to fat were not readily available. A representative sampling of yeast species that were known to produce fat from other substrates was collected. Also, a number of samples that included a wide range of different genera were obtained from natural environments and screened for organisms able to produce fat from whey. Fermenter trials were made with the most successful isolates. These isolates were then identified taxonomically. A more detailed account of the methods follows.

Isolation

Twenty yeasts and molds were obtained from the Northern Regional Research Laboratories (NRRL) in Peoria, Illinois. These yeasts were all species known to be fat producers. Twenty-one cultures were also obtained from the Botany Department, Iowa State University.

Eighteen yeasts and molds were collected from the environment in Ames, Ia. Samples were collected in 50-ml sterile bottles. In a few cases, swabs of material were collected in sterile tubes. Sampling sites and the number of organisms isolated from each were: soil [3], rotting tree stump [2], soil edge of Lake LaVerne [3], horse manure [2], lawn soil [3], spoiled milk [1], standing water [1], and a drain of Iowa State University dairy plant [5]. After preliminary studies indicated that the

university dairy plant furnished promising organisms, 42 isolates were obtained from two other cheese plants in Iowa.

Samples of collected material were streaked (water samples) or sprinkled on potato dextrose agar (Difco, Detroit, Mich.). The medium was acidified to pH 4.5 with tartaric acid to select for yeasts and molds. The plates were incubated for five days at 21C and the colonies observed microscopically for fat production. Fat-producing isolates were purified by three consecutive streaks on potato dextrose agar.

The isolates were screened for their ability to use lactose, grow on whey agar, degrade whey proteins and produce fat.

Screening isolates

Lactose assimilation Cultures were streaked on Czapek's agar medium modified to include 0.1% yeast extract and lactose (15 g/L) instead of the sucrose normally included. Czapeck's is a semisynthetic medium using ammonium oxalate as a nitrogen source. Growth was observed after five days incubation at 21C.

Growth and proteolysis on whey agar Dried whey was reconstituted in distilled water to 6.5% solids. Agar was added at 2.0% (w/v) and the medium prepared for streak plates. Isolates were streaked on this agar and the plates incubated for five days at 21C. The extent of growth and the degree of proteolysis or clearing around the streak was noted.

Lipolysis The lipolysis of fat stained with nile blue sulfate was observed according to the method of Collins and Hammer (19).

Observation of fat Fat was observed in the yeasts and molds as a refractile droplet or was dyed by the addition of a fat stain. The fat

staining Sudan series was tested but found to be inferior while oil red O was found to be an effective stain in most cases. A stock solution of oil red O (Pfaltz and Bauer, Inc., Flushing, N.Y.) was prepared as a saturated solution in 99% isopropyl alcohol. Before use, the solution was diluted with a proportion of 3 parts dye to 2-3 parts water. The undissolved dye was removed by filtration. Wet mounts stained with this solution yielded red fat droplets. For screening purposes, fat production was observed in cultures grown on whey or modified Czapeck's agar.

Identification of Isolates

Identification of the genus and species of these organisms was determined according to the procedures outlined by Lodder (50).

Morphological characteristics were determined in malt extract broth and on corn meal agar (Dalmau plates), yeast morphology agar, and malt agar. These media were observed microscopically every few days for four weeks for morphological characteristics of the organisms. Ascospore formation was investigated on carrot wedges, Gordkowa agar, Klyne's acetate agar, malt extract agar, corn meal agar, and YM agar. These media were examined weekly for six weeks for the presence of yeast producing ascospores.

Physiological characteristics were derived as prescribed by Lodder (50). The organisms were tested for the ethanolic fermentation of six carbohydrates. Assimilation of thirty-four carbon compounds was tested in broth for each organism. The tubes were observed weekly for four weeks for evidence of growth. After four weeks incubation, Gram's iodine was added to the glucose-containing tube to test for the production of

starchlike compounds.

Nitrate utilization was tested by the liquid assimilation test. The nitrite produced by reduction of nitrate and the residual nitrate were tested for by CDC's procedure (Center for Disease Control, Atlanta, Ga., personal communication) using sulfanilic acid and alpha-naphthylamine.

The vitamin and amino acid requirements of the yeasts were determined in Czapeck's semisynthetic agar medium containing either ammonium oxalate (3 g/L) or vitamin-free casamino acids (5 g/L) as a nitrogen source. Amino acid requirements were indicated by poor growth on the ammonium plates and adequate growth on the casamino acid plates. The agar medium was prepared in petri plates and seeded with actively growing washed cells. The vitamins thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxal phosphate, biotin, p-amino benzoic acid and inositol were tested for their growth stimulating ability. Yeast extract was also included as a control.

The solid vitamins were combined into groups of three each and sprinkled on the plate. The zones showing stimulation were examined further by sprinkling the individual vitamins in this group on a second prepared plate. The order of the vitamin placement was altered to reveal multiple vitamin requirements. The plates were examined for zones of growth stimulation indicating a vitamin requirement after three and seven days incubation at 28C.

The yeasts were tested for their ability to grow at high osmotic pressure by streaking on agar plates containing 50 to 60% glucose. The presence or absence of growth was noted after one and two weeks.

The maximum temperature for growth of each organism was determined in a temperature gradient incubator. In this incubator a temperature gradient is maintained in an aluminum block fitted with a heating unit at one end

and a hole for circulating refrigerated water at the opposite end. By placing tubes in the holes bored along the length of the block, incubation temperatures near growth maximums can be selected.

Acid production was determined by the clearing of media containing suspended $CaCO_3$. Urea hydrolysis, lipolysis, arbutin splitting, pigment formation, and gelatin liquefaction tests were also performed (50).

Nutrient Requirements

The aim of this series of experiments was to enhance the rate of growth and fat deposition on whey of selected yeasts that had been successful in fermentation trials. The effects of vitamins, minerals, nitrogen source and lactose concentration were evaluated.

To test growth requirements, the media were prepared by adding the desired nutrients to reconstituted whey. The media were dispensed (50 ml amounts) in 125-ml Erlenmeyer flasks that had been modified to contain interior baffles. These baffles significantly increased the efficiency of aeration and agitation of the medium. The flasks were stoppered with foam disposoplugs (Scientific Products, McGaw Park, Ill.) and autoclaved at 121C for 15 min. The inoculum was prepared by washing a 24 hr potato dextrose agar slant with 10 ml sterile water. One-tenth ml of the suspended cells was aseptically added to each prewarmed flask and incubated on a gyrotary shaker at 28C in a constant temperature room or a constant temperature water bath. For testing vitamins the inoculum was prepared by resuspending the cells on a 24 hr potato dextrose agar slant in 10 ml distilled water. This was transferred to a sterile culture tube and centrifuged. The supernatant was discarded and the pellet was resuspended

in 10 ml distilled water and used to inoculate the flasks. In all tests growth was followed for three days by direct microscopic counting.

Compared with a minimal defined medium for yeasts (62), whey is low in phosphate, Fe, Mn and possibly Mg (86). These nutrients were tested singly and in combination at the following levels per L: K_2HPO_4, 0.1 g; $FeSO_4$, $MgSO_4$ and $MnSO_4$, all at 20 mg. Growth of each organism in these media was compared with a control containing 0.1% yeast extract.

The effect of increased lactose concentrations on growth was tested. Shake flasks containing whey plus lactose at concentrations of 10, 15 and 20% were inoculated and the growth followed for 3 days.

Nitrogen was tested as $(NH_4)_2SO_4$ at 0.005, 0.001 and 0.01 M. Growth was compared with a control containing no additions and also a second containing 0.1% yeast extract.

The vitamins found to stimulate growth on agar plates, as described in the section on yeast identification, were tested in whey cultures. The growth rate and fat production were compared to a control with no additions and a second control containing 0.1% yeast extract.

Requirements for fattening

At the end of the logarithmic growth phase cells were harvested from fermentation medium by centrifugation. They were resuspended in distilled water and centrifuged two times to remove medium constituents. The cells were resuspended in the prepared media at numbers approximating their concentration in the fermenter before harvest (about 6×10^8 organisms/ml). The media and composition were: [1] 4% lactose, [2] 4% lactose, 0.1% K_2HPO_4, [3] 4% lactose, 0.1% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, [4] 4% lactose, 0.1%

K_2HPO_4, 0.1% yeast extract, [5] 4% lactose, 0.1% K_2HPO_4, 0.1% yeast extract and 0.1% NH_4SO_4. Growth and fat production was followed for two days. Fat production was estimated microscopically by measuring the size of the oil drops in the yeast.

Initial fermentation studies

Ten selected yeasts were tested for their ability to grow rapidly and produce fat in fermentation trials. The growth rate, O_2 consumption, CO_2 evolution, COD reduction, fat yields, and fatty acid composition were determined for these one week trial runs. The temperature was maintained at 25C, and the pH was not continuously controlled. Periodically, HCl was added to adjust the pH when it reached inhibitory levels (higher than pH 7.0).

Optimization of Physical Conditions

The pH, temperature, air flow rates and stirring rate for optimal growth and fat production were optimized during batch fermentation runs. When the fermentation vessel was inoculated with logarithmically-growing cells, a short lag period was observed (about three hr) until the cells again began rapidly growing. The rate of cell growth, CO_2 evolution and in some cases O_2 consumption were measured several times over a period of at least 1.5 hr until a uniform rate of increase was observed. Then a small adjustment in a physical condition was made. The usual response was an immediate alteration in the respiration rate and lag in increase in cell numbers. This transition period persisted for about 0.5 hr. When the rate of cell growth and respiration rate stabilized, it was measured

repeatedly for at least 1.5 hr. These data were used to calculate an average respiration rate/cell/hr. Physical conditions were considered optimum when this rate was at a maximum.

Aeration rates were varied with the stirring rate held constant at 500 rpm. Early fermentations showed that this stirring rate gave adequate media mixing.

The pH values were varied between 5.0 and 6.2; however, individual adjustments were only 0.2 to 0.4 units at one time. Larger changes in pH values caused increased lag periods before a steady rate could be observed. The pH values <5.0 or >6.2 were not tested because they strongly inhibited respiration.

The temperature optimization was typically made in changes of two degrees C starting at the cool end of the temperature range. Temperature regulation could be controlled within 0.2C, and a new temperature could be established in 15 min. The temperature was varied ±2.5C from the temperature optimum previously determined in shake flask studies.

When the conditions were optimized for fat deposition during the stationary phase, cell numbers were not increasing or did so only slowly. The transition period was prolonged and the return to a steady respiration rate was more sluggish, so the time for a test adjustment increased about 50-100%.

In some instances the fermentation optimization of physical conditions was repeated in multiple runs. The order of the optimization of physical conditions was changed to signal any possible interactions of the variables

or the importance of the previous immediate history of the cells for the physical optima.

Optimization of Nutrient Conditions

A steady rate of increase of growth, O_2 consumption and CO_2 production was achieved in the fermenter soon after inoculation, as described in the section on physical optimization studies. The aim of nutrient addition was to increase the ability of the medium to support rapid cell division and maximum fat production.

Concentrated solutions of growth nutrients were added to the fermentation medium via the inoculation port. After an addition, observations were made on rates of cell numbers increase and CO_2 evolution for a period of about 1.5 hr. If no changes occurred, it was concluded that the added nutrient was not in limiting concentration in the fermentation medium. Correspondingly, if a rate increase was observed, the nutrient was considered rate limiting. When a stimulatory nutrient was added, typically, a rapid rate increase was observed within 15 min in logarithmic growth and 0.5 hr in the stationary phase. The new growth rate and CO_2 evolution would be observed for a short period and then, when the added nutrient was exhausted, the previous rate would be observed. An excess of this stimulating nutrient was added. The concentration needed was calculated from the cell number at the time of testing and the maximum cell numbers expected in the fermenter. For example, if an amount of nutrient gave maximum stimulation when the cell counts were 5×10^6/ml and a 1×10^8/ml population was expected, 100x the stimulatory amount was added. This afforded an excess of the limiting nutrient.

When a nutrient stimulated oil deposition in the stationary phase after maximum cell numbers had been reached, it was added in 10x the stimulating concentration.

After a limiting nutrient had been identified and added to the fermenter in excess of anticipated needs, a 1.5 hr period of rate change stabilization was allowed. Then a search for another stimulatory nutrient was begun.

Residual COD Evaluation

After the conditions had been optimized for rapid growth and maximum fat production, fermentation under these conditions was evaluated for COD reduction.

An attempt was made to determine the composition of the residual COD and also why this residual was not further reduced in the process of fermentation.

The presence of an inhibitory factor or the lack of an essential growth nutrient which caused the fermentation of the whey medium to be only partially complete was investigated in the case of Candida curvata R and Trichosporon cutaneum 24.

The medium was removed from the fermenter at the end of the fermentation, i.e., when the rate of CO_2 evolution began to drop rapidly. The cells were removed by centrifugation and the spent medium dispensed into 125-ml shake flasks. Whey was added at 0, 10, 20 and 50% of the spent medium. In addition, a spent medium plus 0.1% yeast extract, and 0.1% yeast extract and 1% lactose was prepared. This series was designed to indicate the ability of spent medium plus whey as a source of vitamins and

minerals to support growth. The growth and fattening was compared with a control containing no additions and a whey control.

Whey Permeate Studies

Fermentation runs were also performed using whey permeate (proteins removed) as a substrate. Optimum nutrient and physical conditions were assumed to be the same as in the whey fermentations. The permeate was reconstituted to a COD of about 65,000 mg/L corresponding to a lactose concentration of about 5.7%. Fermentation mass balance equations were more easily determined on this medium as the interference of whey proteins was removed.

The ability of different concentrations of whey permeate to support growth and fattening of yeasts was determined.

Whey permeate was condensed in a rotary vacuum evaporator to a 50% lactose concentration. This was diluted to prepare culture media containing 5%, 20%, 35% and 50% lactose. Flasks were inoculated and the growth and fattening followed for 20 days. The preparation of the medium, inoculum and incubation conditions were the same as those used to study vitamin and mineral additions to whey.

Mass Balance Calculations

The total mM CO_2 produced and mM O_2 consumed during the fermentation was calculated by integrating the plots of mM/hr/10 L versus time with a compensating polar planimeter. These values were used to estimate the amount of lactose used for energy and for fat production.

Assume:

1. Complete respiration of lactose by cells for energy must occur by

$$C_{12}H_{22}O_{11} + 12O_2 \longrightarrow 12CO_2 + 11\ H_2O.$$

2. Production of fat from lactose occurs by the degradation to acetyl-CoA units

$$C_{12}H_{22}O_{11} \longrightarrow 4 \text{ acetyl-CoA} + 4CO_2.$$

If the amount of CO_2 and O_2 are measured, it is possible to calculate the total amount of lactose used

$$\text{total lactose} = \text{lactose respired} + \text{lactose to fat.}$$

$$\text{mM total lactose} = \frac{mMO_2}{12} + \frac{mMCO_2 - mMO_2}{4}.$$

3. Assume that the triglyceride produced is a triacylglyceride composed of three hexadecanoic acids (or palmitic acids) esterified to one glycerol

$$C_{12}H_{22}O_{11} \longrightarrow 4 \text{ acetyl-CoA} + 4CO_2$$

$$4 \text{ acetyl-CoA} \longrightarrow 1 \text{ octanoic acid}$$

$$1 \text{ octanoic acid} \longrightarrow 1/2 \text{ hexadecanoic acid}$$

$$1/4\ C_6H_{12}O_6 \longrightarrow 1 \text{ glycerol}$$

$$6\ 1/4\ M\ (C_{12}H_{22}O_{11}) \longrightarrow 1 \text{ tripalmitin.}$$

4. From the molecular weight of tripalmitin (807.36 g/M), the g of fat produced/10 L medium can be calculated using the total mM CO_2 and mM O_2/10 L medium.

$$\text{g fat/10 L} = \frac{(\text{mM } CO_2 - \text{mM } O_2)(807.36 \text{ g/M}) \times 10^{-3} \text{ M/mM}}{4 \times 6.25}.$$

These calculations assume that all of the CO_2 produced that doesn't come from respiration comes from fat production. This assumption will overestimate the amount of fat produced, because many other cell components, particularly amino acids, will be produced with CO_2 liberation.

The lactose estimated used by these calculations will be underestimated. Lactose can be used in many biosynthetic reactions, such as cell wall formation and some amino acid syntheses, that would not liberate CO_2.

RESULTS AND DISCUSSION

Culture Selection

The search for fat-producing strains that would grow on whey, among species reported to produce fat on other media, was only marginally successful. Of the cultures obtained from NRRL, only six produced fat on Czapek's or whey agar (see Appendix Table 12 for screening results).

The screening procedure was designed to indicate additional desirable or undesirable characteristics. For example, two of the fat producers were weakly proteolytic indicating that they might be able to use whey proteins for a nitrogen source. One of the fat producers, G. candidum, was highly lipolytic indicating a possible problem in isolating and refining its fat. Another fat producer, Endomyces vernalis, was eliminated because it grew very slowly on whey agar or whey agar plus 0.1% yeast extract.

Only one of the cultures, Mycogne sp., obtained from the culture collection of the Iowa State University Botany Department produced fat (Appendix Table 13). This organism grew very slowly on whey agar and was eliminated from further study. Fat production seems to be a somewhat rare occurrence in yeasts and molds that will grow on whey agar. Moreover, growth was delayed on lactose-containing media compared to malt agar slants.

Theoretically, many strains from the same fat-producing species may also be fat producers. Only one strain from each species was evaluated, but it seems likely that fat production may be strain specific, a suggestion that is supported by the work of several authors on fat production by

different strains of S. cerevisiae and Lipomyces starkeyi (22). Possibly examination of additional strains of the known fat-producing species would have turned up some that were successful on lactose; however instead an effort was made to isolate suitable fat-producing yeasts and molds.

Environments were chosen for sampling that would exert some selective pressure for organisms able to use lactose and produce fat. Since milk is the major natural source of lactose, the flora of the Iowa State University cheese plant was sampled. Emphasis was placed on sampling floor drains and other areas receiving intermittent milk additions. A wide range of other environments were also sampled (see Appendix Table 14 for screening results).

Only three of the organisms, all isolated from the Iowa State University dairy plant, produced fat and assimilated lactose. One was lipolytic and two were weakly proteolytic. They all grew very rapidly on whey and modified Czapek's agar and produced luxurious growth in 24 hr.

The successful isolation of these organisms from the Iowa State University cheese plant encouraged the sampling of two other Iowa dairy plants. Of the 42 isolates examined, 14 produced fat and used lactose. None of the fat producers were proteolytic. These 14 isolates were placed into four groups based on the original source of isolation and morphological characteristics. Organisms were selected from each group that grew rapidly and produced the most fat on whey agar. These organisms were saved for further screening.

Initial Fermentation Studies

The ten most successful organisms selected from initial screening were tested for their ability to grow and produce fat in one week whey fermentation trials. Results for seven of these organisms are presented in Table 2. Results for G. candidum NRRL 7366, L. lipofer NRRL Y-1394, and R. glutins NRRL Y1091 are not presented because their fermenter trials were terminated early. The latter two organisms grew poorly in whey and did not produce observable fat after about 60 hr. G. candidum NRRL 7366 grew well but did not accumulate fat. These results were somewhat unexpected, as agar cultures had produced fat after extended incubation. Species of G. candidum had been used successfully in whey by others (90) and L. lipofer had been reported (22,75) to accumulate fat when grown on lactose. Most of these reports used extended incubation periods, e.g., up to 33 days in the case of L. lipofer (22), and therefore may not be expected to compare well with the fermenter studies.

Table 2 shows that L. starkeyi and organism 4 grew poorly and produced little fat. Yeast extract stimulated L. starkeyi at 10 g/10 L, but not at 1.0 g/10 L. This suggests that at the higher levels yeast extract is serving as an energy source.

Isolate B was comparable to isolate 40 in the fermentation trials, but grew to lower cell numbers indicating possible nutrient limitations. Isolate 4 was eliminated from further consideration because of poor growth and limited fat production.

The four isolates R, D, 24 and 40 were more successful on the basis of fat formation and maximum cell numbers. From 10 L of whey, isolates

Table 2. Characteristics of some initial whey fermentations with selected yeasts.

Organism	mM CO_2 total	mM O_2 total	M lactose used	Residual COD	g fat/10 L produced	Log max cell no.
40 (*T. cutaneum* 40)	1715.46	815.4	0.292	48,000	29.02	8.34
4	470.00	271.3	0.073		6.41	8.20
24 (*T. cutaneum* 24)	3375.00	491.9	0.762	25,000	92.95	9.30
B	1770.00	903.6	0.291		27.94	7.25
D (*C. curvata* D)	5311.38	2008.8	0.993	28,000	106.47	9.07
R (*C. curvata* R)	5394.38	1586.2	1.084	15,000	122.77	9.20
L. starkeyi	374.00	151.5	0.068		7.17	7.56

R and D both used about 0.9 to 1.1 M of lactose, produced about 106-123 g of fat, and grew to maximum cell concentrations of 1.0×10^9 and 1.6×10^9/ ml respectively.

Analyses of the spent medium collected at the end of the fermentation trials for each of the four organisms, R, D, 24 and 40, indicated that the COD was reduced 21% to 76%, from an initial value of 62,000 mg/L.

Isolates R, D, 24 and 40 were believed to be the four best organisms for further study. They all grew rapidly, did not appear to require nutrients not already present in whey, and produced fat. They all raised the pH of the whey during the early part of the fermentation, possibly indicating lactic acid utilization or ammonia production from deamination of amino acids.

It is possible that the isolates (e.g., isolate B) rejected at the end of fermentation trials could have been more successful fermenters if additional limiting nutrients had been supplied. However, this would have increased fermentation costs. It could be argued also that optimum physical conditions were not used in this survey resulting in decreased yields, but these conditions would be expected to affect the metabolic rate rather than the maximum cell number.

Identification of Isolates

The four isolates selected from fermenter studies were taxonomically identified. Isolates R and D were very similar in general morphology. They both reproduced primarily by budding, produced some pseudomycelium on corn meal agar, and more abundant true mycelium on water agar and in malt extract broth. Heavy blastospore formation from true hyphae, typical of

the genus Candida, was observed with both isolates on yeast morphology agar, but was more abundant for isolate R than for D. True hyphae were produced only after about two weeks incubation and were more common within the agar than on the surface. Growth in malt broth after two days was scant, but a few budding cells were found. After one month a thin, tan pellicle and ring were formed with a little sediment in the bottom of the tube. No sexual spores of any kind were observed, nor were chlamydiospores or other asexual resting spores.

The physiological test results for both of these organisms are found in Table 3. The striking characteristic of these organisms is their lack of fermentative ability and broad assimilative capacity. The physiological and morphological characteristics of these organisms are very similar to Candida curvata (50) differing only slightly from the type species. Additional characteristics determined for these isolates include growth in 50% and 60% glucose, lipolytic ability, gelatin liquefaction, and weak acid production by isolate D. These two organisms differ in their morphological and physiological characteristics. Isolate R formed much more abundant true hyphae and balistospores than did isolate D. Secondly, they differed in their maximum growth temperature, assimilation of ribitol and production of acid from glucose. Thus, these organisms are believed to be two different strains of C. curvata and will be referred to as C. curvata R and C. curvata D.

The imperfect genus Candida is a very heterogenous group. From time to time a perfect or sexual phase of the life cycle may be discovered for a species and then it is transferred to a perfect genus in the Ascomycetes

Table 3. Taxonomic characteristics of the type species of C. curvata and strains R and D.

Test	C. curvata		
	R	D	Type species
Fermentation	Negative	Negative	Negative
Carbon assimilations			
D-glucose	+	+	+
D-galactose	+	+	+
L-sorbose	-	-	+ or -
salicin	+	+	+
sucrose	+	+	+
maltose	+	+	+
melibiose	-	-	-
cellobiose	+	+	+
trehalose	+	+	+
lactose	+	+	+
raffinose	+	+	+
melezitose	+	+	+ or -
inulin	-	-	-
starch	+	+	+
xylose	+	+	+
D-arabinose	-	-	+ or -
L-arabinose	+	+	-
D-ribose	+	+	+
L-rhamnose	+	+	+
ethanol	+	+	+
glycerol	+	+	+
erythritol	-	-	+
ribitol	+	-	+
galactitol	-	-	-
D-mannitol	+	+	+ or -
sorbitol	+	+	+ or -
m-inositol	+	+	+
lactic acid	+	+	+
succinic acid	+	+	+ or -
citric acid	+	+	+ or -
glucosamine	+	+	N.G.[a]
pyruvic acid	-	-	N.G.
glucono-δ-lactone	+	+	N.G.
Nitrogen assimilations			
$NO_3^=$ or NO_2^-	-	-	-

[a]N.G. = test result not given by Lodder (50).

Table 3. Continued.

Test	C. curvata		
	R	D	Type species
Vitamin requirement	Thiamine	Thiamine	Biotin + thiamine
Growth on 50% glucose	+	weak	N.G.
60% glucose	-	-	N.G.
Maximum growth temperature	37C	35C	35-38C
Starch formation	-	-	+
Urea hydrolysis	+	+	+ or -
Arbutin splitting	-	-	N.G.
Lipolytic	+	+	N.G.
Gelatin liquefaction	+	+	N.G.
Acid production from glucose	-	weak	N.G.

or Basidiomycetes. But this rarely happens, and some mycologists have found a more useful tool for grouping these imperfect forms by means of DNA base ratios (in moles % guanine + cytosine). This information also can be used to determine which perfect group a yeast might belong to, since Myxomycetes and Phycomycetes have a guanine + cytosine percentage less than 50%, the Ascomycetes near 50% and the Basidiomycetes more than 50% (56). On this basis C. curvata has been placed in the humicola group along with C. heveanensis, C. muscorum and C. scotti (56). These organisms all have guanine + cytosine percentages of about 60% and are closely related to the genus Leucosporidium of the Ustilaginales and Basidiomycetes.

The type species of C. curvata was originally isolated from sputum. It was originally considered a variety of C. heveanensis, but this latter organism was reclassified as Cryptococcus laurentii in 1952 by Lodder (50). The curvata variety was raised to species rank and named Candida curvata (50). It has been isolated from cattle (50) and soil (79) but appears to have been studied little.

The isolates 24 and 40 were identified as strains of Trichosporon cutaneum. These two strains are distinct morphologically and physiologically (Table 4). Isolate 24, hereafter referred to as Trichosporon cutaneum 24, buds to produce cells of irregular size and shape and abundant true hyphae which breaks up into arthrospores. These spores are often formed in the middle of a hyphal strand. They appear highly refractile, dark and more granular with age. Segments of empty hyphae may precede a chain of arthrospores. Arthrospore formation was abundant after one to two weeks on corn meal agar and carrot sticks.

Table 4. Taxonomic characteristic of the type species of T. cutaneum and strains 40 and 24.

Test	T. cutaneum		
	24	40	Type species
Fermentation	Negative	Negative	Negative
Carbon assimilation			
D-glucose	+	+	+
D-galactose	+	+	+
L-sorbose	-	+	+
salicin	+	+	+ or -
sucrose	+	+	+
maltose	+	+	+
melibiose	+	-	+ or -
cellobiose	+	+	+
trehalose	+	+	+
lactose	+	+	+ or -
raffinose	+	-	+ or -
melezitose	+	+	+ or -
inulin	+	-	-
starch	+	+	+
xylose	+	+	+
D-arabinose	+	-	+
L-arabinose	+	+	+ or -
D-ribose	+	+	+
L-rhamnose	+	+	+ or -
ethanol	+	+	+
glycerol	+	+	+ or -
erythritol	+	-	+ or -
ribitol	+	-	+ or -
galactitol	-	+	+ or -
D-mannitol	+	+	+ or -
sorbitol	+	-	+ or -
m-inositol	+	+	+
lactic acid	+	+	+ or -
succinic acid	+	+	+ or -
citric acid	+	+	+ or -
glucosamine	+	+	N.G.[a]
pyruvic acid	-	+	N.G.
glucono-δ-lactone	-	+	N.G.
Nitrogen assimilations			
$NO_3^=$ or NO_2^-	-	-	-

[a] N.G. = test result not given by Lodder (50).

Table 4. Continued.

Test	*T. cutaneum*		
	24	40	Type species
Vitamin requirement	Thiamine	Thiamine	Thiamine
Growth on 50% glucose	+	+	-
60% glucose	+	+	-
Maximum growth temperature	35C	35C	29-41C
Starch formation	+	-	+ or -
Urea Hydrolysis	+	+	+ or -
Arbutin splitting	+	-	N.G.
Lipolytic	+	+	N.G.
Gelatin liquefaction	+	+	N.G.
Acid production from glucose	+	+	N.G.

In stationary broth cultures budding occurred and hyphae were formed. A rough, undulating cream colored pellicle, but no sediment, was observed after one month in malt broth. In aerated whey cultures, cells reproduced by budding on a very broad base and then separated by a snapping action similar to the 'snapping division' observed in the bacterial genus Arthrobacter.

Ascospores, teliospores, balistospores, endospores or chlamydiospores were not observed, nor were clamp connections seen. In one instance clavate conidialike structures were observed on water agar. The significance of these structures isn't known.

The carbohydrate assimilation tests and other physiological test results (Table 4) agree with those described for this species by Lodder (50) except for the utilization of inulin by this organism. Additional physiological characteristics determined for this isolate include: fat splitting +, growth on 50 and 60% glucose, production of acid from glucose, complete hydrolysis of gelatin and splitting of arbutin. In addition, the culture had a faint musty odor and produced amyloid compounds. The optimum temperature for growth was 28C and the maximum 35C.

Isolate 40, referred to hereafter as Trichosporon cutaneum 40, was very similar to T. cutaneum 24 (Table 4). Budding cells and pseudomycelium were abundant on corn meal agar after one week. Unusually shaped grainy cells were often observed. After three weeks true hyphae that produced intercallary arthrospores were common. These spores were often grainy, not truly refractile and contained fat droplets. The arthrospores that were formed were often slightly larger than the hyphal strand they were

derived from. The hyphae preceding an area of arthrospore formation was definitely full of protoplasm, but cross walls were infrequent. But in other cases, an entire hyphal strand would break up into many arthrospores side by side. Arthrospore formation was most abundant after three weeks on corn meal agar. Other types of spores whether sexual or asexual were not produced.

In stationary broth cultures budding was common but true- and pseudohyphae were occasionally produced. A fragile, smooth, white pellicle and abundant sediment was present in malt broth after three weeks.

The carbohydrate assimilation tests and other physiological test results agree with those for the type strain of T. cutaneum except for this strain 40's ability to use inulin. This organism was tested for several physiological characteristics not mentioned by Lodder (50) in the species description. The organism was lipolytic, produced a potatolike odor, grew in 50 and 60% glucose, produced acid from glucose, hydrolyzed gelatin, and was unable to split arbutin. The maximum temperature for growth was 35C and the optimum was 33C. This isolate did not produce amyloid compounds.

Trichosporon cutaneum is a well-known organism widely distributed in nature. It has been identified as the causative agent of piedra, a disease of hair follicles in mustaches and beards (50). It has also been frequently isolated from water and sewage (1). T. cutaneum, an imperfect yeast, may be closely related to the Basidiomycetes having a similar DNA base composition (guanine + cytosine). However, sexual spores characteristic of the Basidiomycetes haven't been observed in this genera.

Optimization of Physical Conditions

It is assumed that if a physical parameter is changed during the fermentation that the beneficial or detrimental character of the change can be judged by an increase or decrease of the growth or respiration rate. This method of optimization has been used previously in continuous fermentations (34,55,77).

A summary of the optimum physical conditions for growth and fattening is found in Table 5. Figure 3 shows the effect of temperature. The temperature optima for these organisms had been determined to be in the range of 28 to 32C from growth in shake flasks incubated at 25, 28, 32, and 35C. Temperature gradient incubator studies indicated that the maximum growth temperatures were 37C for C. curvata R and 35C for the other organisms. Since the maximum growth temperature is often about 5C above the optimum, the optimum temperature of these organisms would be expected to be near 30C. No differences were found in the optimum fattening and optimum growth temperatures.

Studies of pH optimization of Penicillium chrysogenum (5) showed that respiration rate changed parabolically with pH. The data from these optimization experiments (Figure 4) appear to follow the same general trend. The pH optima for fattening are, in all cases, different than those for optimum growth. It is not unusual to find pH optima differences for growth and product formation (64). Such a difference has been reported for growth and fattening in R. gracilis (45).

Aeration rates are often assumed to be adequate for aerobic cultures when the O_2 demand does not exceed the supply. It is often sufficient to

Table 5. Optimization of physical conditions in fermentation trials.

Organism	Physical condition					
	Logarithmic growth			Fattening phase		
	pH	Temp.[a]	Aeration[b]	pH	Temp.[a]	Aeration[b]
C. curvata R	5.8	30	2.5	5.2	30	>7.0
C. curvata D	5.4	28	2.5	5.8	28	>7.0
T. cutaneum 24	5.4	28	2.5	5.8	28	>4.0
T. cutaneum 40	5.7	33	2.5	5.6	33	>4.0

[a]Temp. = Temperature in C.

[b]Aeration = L air/min/10 L medium.

Figure 3. CO_2 production rate of yeasts during the growth phase at different temperatures. □ C. curvata R, ● C. curvata D, △ T. cutaneum 24, ○ T. cutaneum 40.

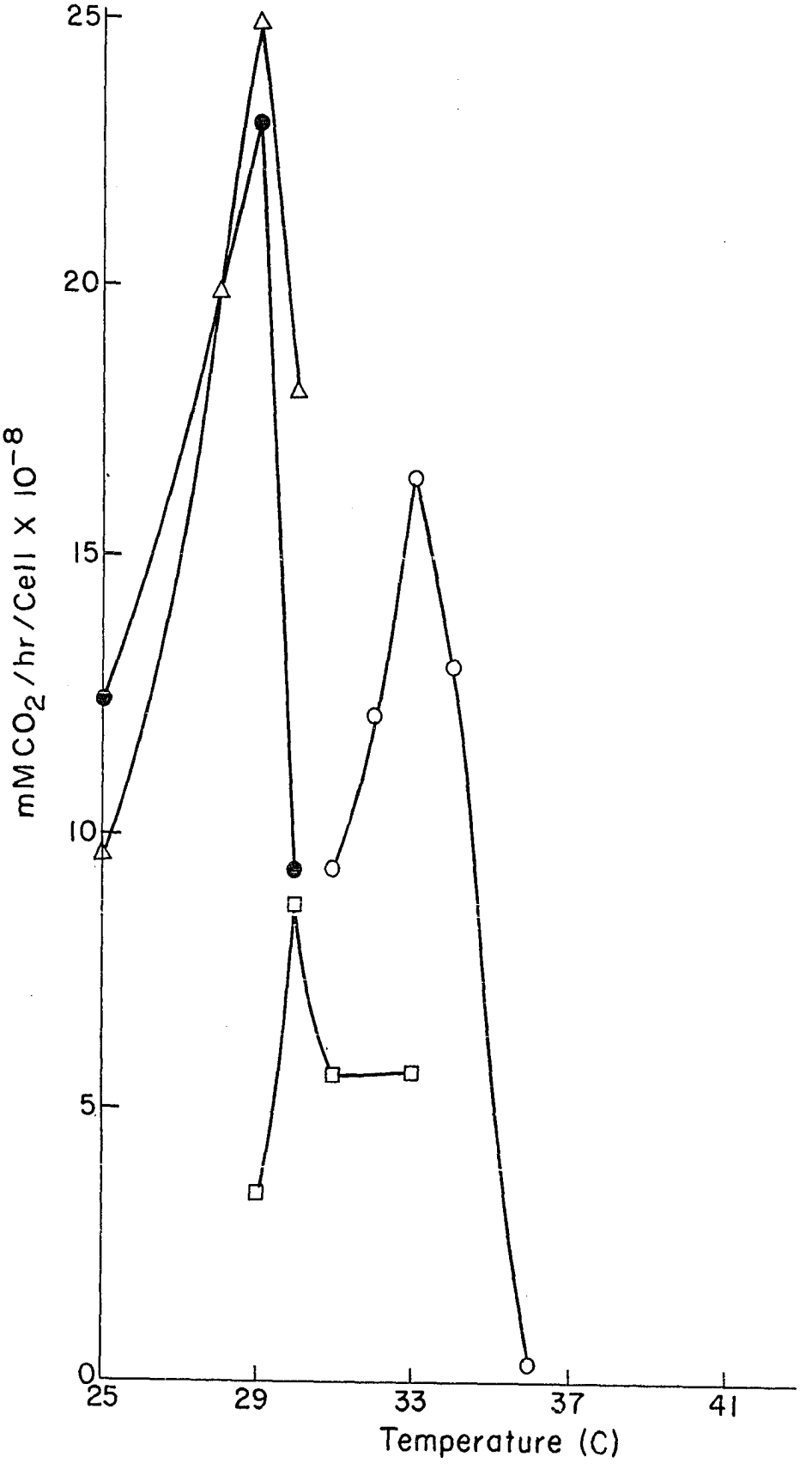
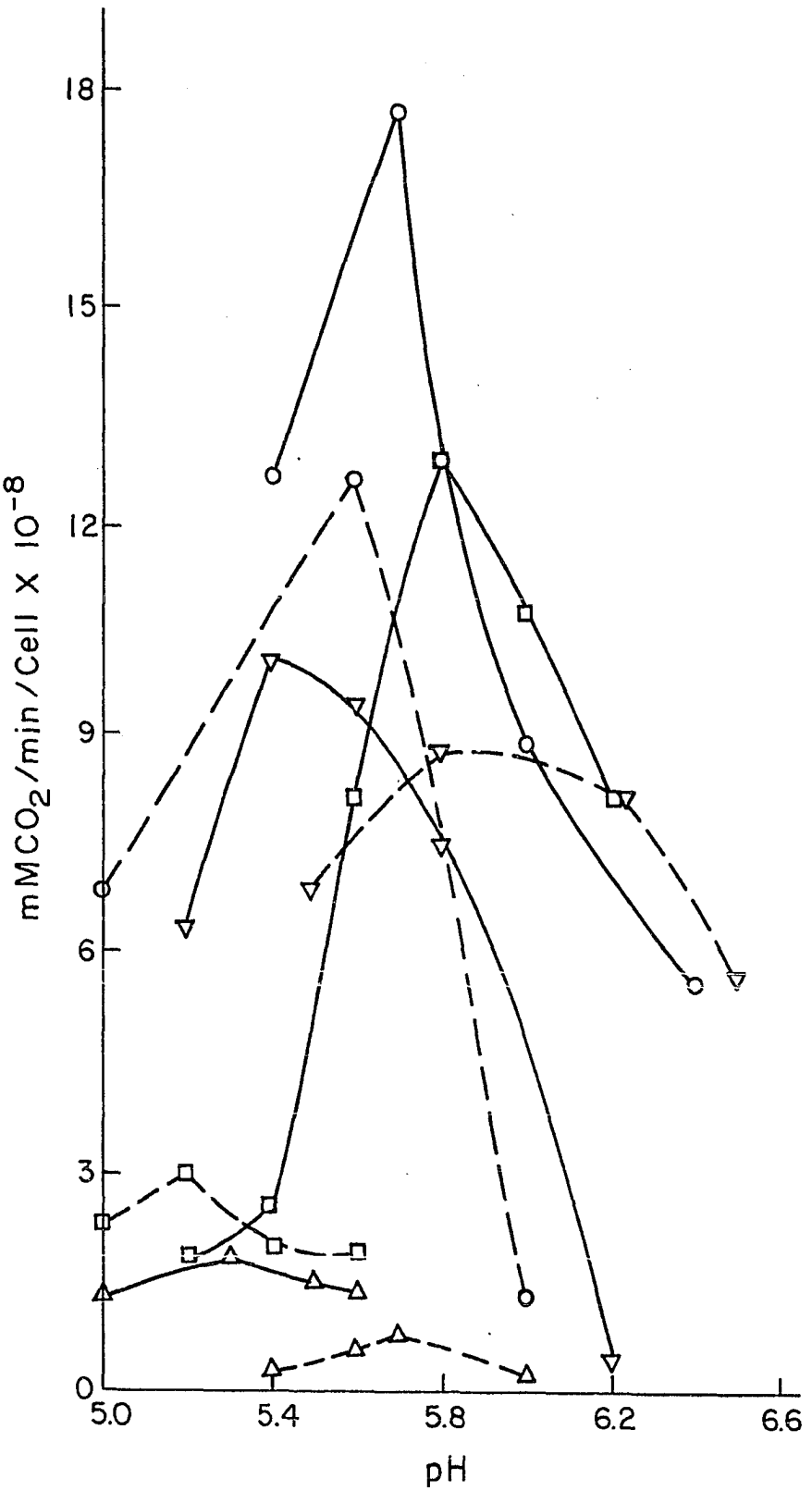

25
20
15
10
5
0
mM CO2 /hr /Cell X 10-8
25
29
33
37
41
Temperature (C)

Figure 4. CO_2 production rate of yeasts during the growth and fattening phases as a function of pH. —— During logarithmic growth, --- during fattening. □ C. curvata R, ▽ C. curvata D, △ T. cutaneum 24, ○ T. cutaneum 40.


18
15
12
9
6
3
0
mMCO2/min/Cell X 10-8
5.0
5.4
5.8
6.2
6.6
pH

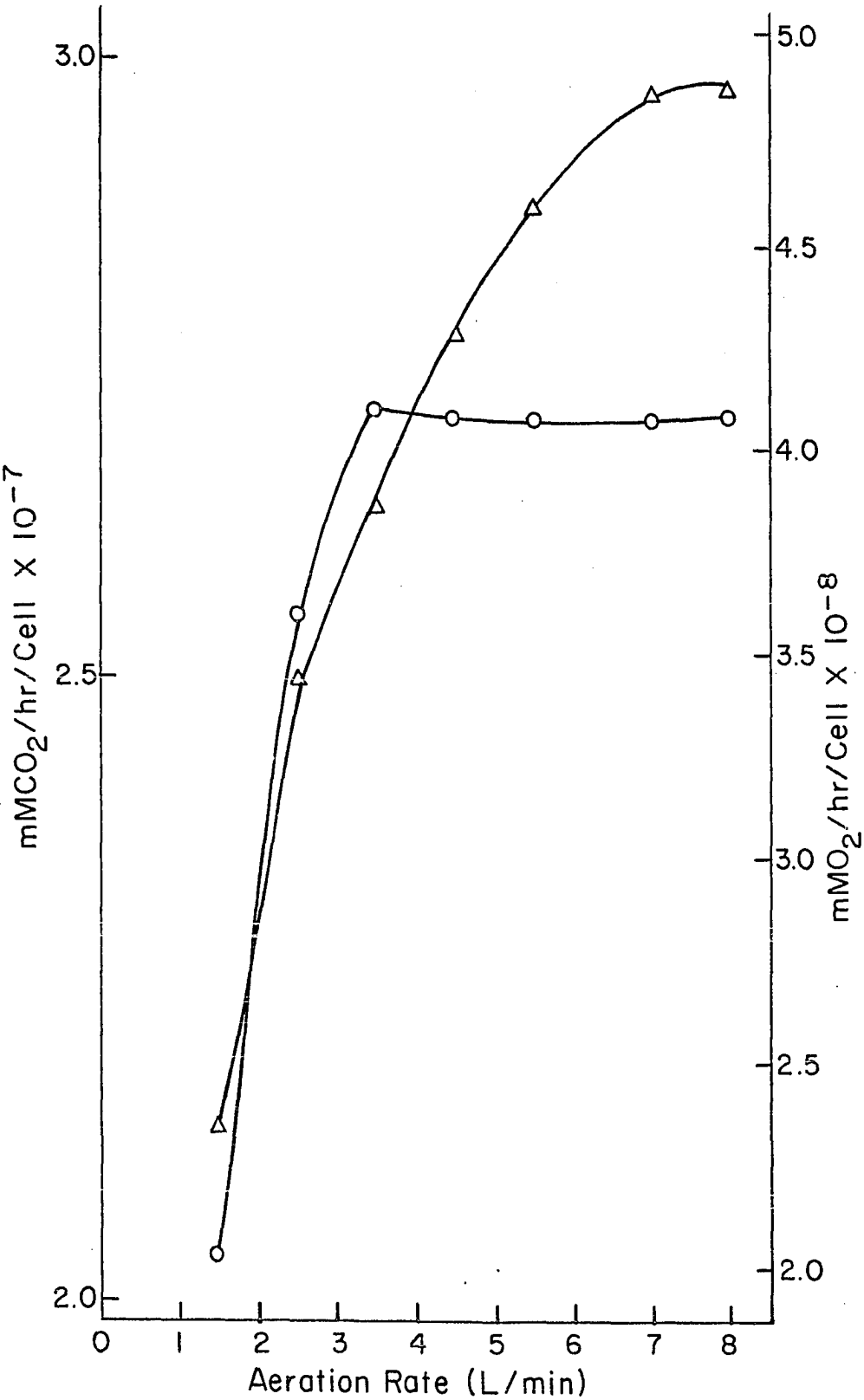
choose aeration rates slightly above the demand so that O_2 will not become limiting at any time during the growth (11). In fermentations using these four organisms, a rate of 2.5 L/min/10 L medium maintained dissolved O_2 levels above the maximum demand. When higher cell populations were reached in later fermentations, an adjustment in aeration rate was made. This was done by switching to the higher aeration rates required for fattening before the fattening phase was actually reached.

The optimum aeration rates for fattening were higher than those for growth. Maximum oxygen utilization during fattening occurred at aeration rates of 3 L/min/10 L in a study of C. curvata (Figure 5). However, maximum CO_2 production was observed at an aeration rate greater than 7 L/min/10 L. This trend in aeration demand was observed during the fattening phases for the other three yeasts and aeration intensities were maintained at 10 L/min/10 L medium during fattening for all organisms. This rate was selected rather than 7 L/min/10 L because of slightly higher cell numbers in later fermentations. The stimulation may be due to the removal of toxic end products of metabolism by the air. This effect has been observed by others (18).

Optimization of Nutrient Conditions

The nutritional requirements of these organisms were determined on whey agar and in shake flasks. While some important trends in nutritional requirements in whey were established, it soon became apparent that many of the changes in growth rate or maximum cell numbers were small and difficult to evaluate. These small changes could be more readily evaluated in fermentations where physical conditions could be rigorously controlled,

Figure 5. CO_2 evolution and O_2 utilization of C. curvata R at different rates of aeration. ΔCO_2, $O O_2$.


3.0
2.5
2.0
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
1
2
3
4
5
6
7
8
Aeration Rate (L/min)
mMCO2/hr/Cell X 10-7
mMO2/hr/Cell X 10-8

but, some of the general characteristics of these shake flask studies will be briefly summarized here.

The vitamin requirement of these four organisms had been previously determined in taxonomic studies. Thiamine was stimulatory for all on minimal media. In addition, inositol was stimulatory for both strains of C. curvata and T. cutaneum 24. When these vitamins were added singly and in combination to whey shake flasks, no growth stimulation was observed and they were inhibitory for C. curvata R. Yeast extract (0.1% w/v) additions increased cell numbers 2x higher than the control for the two strains of C. curvata and T. cutaneum 24, but not for strain 40. Abnormal cell morphology was observed when yeast extract was added to strain 40, but not the other organisms.

Amino acids were not required by three of these yeasts. The growth of T. cutaneum 24 was slow on agar medium lacking amino acids and containing ammonium oxylate as a nitrogen source. However, good growth was observed on this medium in the region of yeast extract addition. This indicated a preference for preformed amino acids or perhaps several nutrient requirements.

Inorganic nitrogen additions to whey stimulated the growth of these four organisms. The addition of ammonium sulfate to whey (at 14 mg N/ 100 ml) increased the cell numbers of strain R, from 5.9×10^8/ml for the control, to 1.8×10^9/ml. C. curvata D showed a similar stimulation from 4.8×10^8 to 1.3×10^9 cells/ml when 1.45 mg nitrogen were added as ammonium sulfate. For T. cutaneum 40 this nitrogen addition also increased the number from 2.9×10^8 to 6.4×10^8/ml, and for T. cutaneum 24 from 3.2×10^8

to 7.2 x 10^8/ml. Since yeast extract is approximately 10% nitrogen (70) a 0.1% addition would be 10 mg N/100 ml. This amount of yeast extract caused about 50% increase in cell numbers while 14 mg N/100 ml caused a somewhat larger increase. The primary effect of yeast extract addition was assumed to be due to its nitrogen content.

Metal salt additions to whey cultures showed slight stimulations of growth rates and increases in maximum cell numbers in some cases. But when these salts were added in combinations, growth inhibition was often observed and cells grew in bizarre, long filaments without cross walls. The metal content of yeast extract may have been responsible for the unusual morphology of T. cutaneum 40 mentioned earlier. Mineral requirements were evaluated more fully in fermentation trials.

Nutritional Optimization-Fermenter Studies

C. curvata R

When 2.0 g/10 L of yeast extract were added to C. curvata R (under optimum physical conditions), the cell numbers increased from 5.1 x 10^8 to 1.1 x 10^9/ml. This was accompanied by a production of about 1000 mM CO_2. The effect of yeast extract was believed to be due to nitrogen, but Mn as well as other metal salts were stimulatory to growth in whey shake flask studies. The effect of metal salts and nitrogen were further evaluated in the fermenter.

When 25 mg Mn (as $MnSO_4$) were added to the fermenter an increase of numbers from 5.1 x 10^8 to 7.8 x 10^8/ml was observed. Further Mn additions had no effect on growth or CO_2 production. When 1.28 g nitrogen (as 10 ml 15.5% NH_4OH) were added to the fermenter the population showed another

increase from 7.8×10^8 to 1.1×10^9/ml and resulted in 946 mM CO_2 being produced. Addition of 53 mg phosphorous (as KH_2PO_4), previously shown to be stimulatory in shake flasks, showed another slight increase in cell numbers to 1.3×10^9/ml. Other nutrients, such as Mg, Fe, thiamine, pyridoxal, adenine, guanine, uracil, and cytosine which were present in yeast extract but not known to be stimulatory, were also added and had no effect on CO_2 production.

The primary effect of yeast extract on growth was attributed to the nitrogen, phosphorous and Mn content which is 190 mg, 20 mg, and approximately 1.0 mg/2.0 g yeast extract, respectively (70). None of these nutrients affected CO_2 production without also affecting growth.

When the rate of cell growth and CO_2 production started to decline at the end of the fermentation, yeast extract was added to check the possibility that nutrients were then limiting growth or CO_2 production. The rate of CO_2 production continued to fall and cell lysis began which resulted in a 25% reduction in cell numbers.

C. curvata D

Shake flask studies of C. curvata D indicated that the addition of ammonium sulfate (14 mg N/100 ml) stimulated growth markedly from 4.8×10^8 to 1.4×10^8/ml. The effect of yeast extract addition on growth was very slight. In the fermenter, yeast extract (200 mg) additions did not show any stimulation, but when 3 ml of concentrated NH_4OH (15.5%) were added at the beginning of fermentation, numbers reached 7.0×10^8 as compared with 5.3×10^8 in earlier trials. Salts such as $MnSO_4$, $MgSO_4$ and K_2HPO_4

had no effect and may have been slightly inhibitory. Seemingly the nutrients present in whey were sufficient for growth when NH_4OH was added.

T. cutaneum 40

In shake flask studies with T. cutaneum 40, nitrogen and $MgSO_4$ stimulated the growth rate slightly. Other nutrient additions had no effect. The growth in whey was relatively poor, reaching only about 1.9×10^8/ml. Similar populations were also reached in the physical optimization studies, and this resulted in very long fermentation times.

An unusual feature of the growth pattern of this organism which affected the nutrient optimization studies was the diphasic nature of the CO_2 production. After cell numbers had reached their maximum, the rate of CO_2 production fell off very rapidly. Then the rate increased and reached a maximum while fat was being produced. If nutrients were added between these two phases they had little effect. But when yeast extract was added during logarithmic growth or in the fattening phase numbers increased.

In other organisms the stimulatory action of yeast extract had been duplicated by nitrogen and metal salts, so fermenter trials were conducted with 10 ml concentrated NH_4OH/10 L added at the start. The cells reached slightly higher maximum densities of 2.2×10^8/ml compared with 1.9×10^8/ml in previous trials. When 350 mg $MgSO_4$ were added, the population increased slightly to 2.8×10^8/ml. When 250 mg of yeast extract were added numbers increased to 3.8×10^8 cells/ml. A second fermentation trial revealed that the stimulation of yeast extract would be duplicated by the addition of 550 mg $MgSO_4$ and 11 ml NH_4OH (or 1.41 g N). The

population reached 6.0×10^8/ml. Other metal salts, casamino acids, yeast extract, or additional nitrogen had no effect on CO_2 production or cell numbers.

T. cutaneum 24

Shake flask studies showed that yeast extract stimulated growth about twofold. These studies also indicated that addition of Fe or phosphate stimulated growth rate, but not maximum numbers, while the addition of Mn or Mg resulted in higher maximum cell numbers.

When 100 mg $FeSO_4$ were added to the fermenter during the early logarithmic phase of growth, an increase in CO_2 production rate was observed and this was associated with a slight decrease in generation time. Additions of more than 100 mg $FeSO_4$ had no effect on CO_2 production or generation time. $FeSO_4$ had no effect on final cell population. The addition of 5 mg each of $MgSO_4$ and $MnSO_4$ at the end of logarithmic growth resulted in an increase in cell numbers from 1.8×10^8 to 5.9×10^8/ml. Further additions (15 mg each) of these salts in stationary phase had a slight stimulatory effect on CO_2 production (about 35.0 mM CO_2) but not on cell numbers. After the addition of 20 mg $MnSO_4$ and 30 mg $MgSO_4$, yeast extract gave no further stimulation.

In shake flask studies populations had reached 7.2×10^8/ml when ammonium sulfate was added. When 10 ml NH_4OH (15.5%) and Mg and Mn salts were added to the whey at the beginning of the fermentation, maximum cell numbers reached 1.1×10^8/ml compared with 5.9×10^8/ml with Mg and Mn salts alone. Casamino acids, vitamins, glucose or other salts such as

K_2HPO_4 added in small amounts had no effect on cell numbers or CO_2 production rate.

After the cells had been in stationary phase for several hours, additional nutrients stimulated CO_2 production but not growth. The nutrients may have stimulated fat production only, or been insufficient for increased growth. The cells may also have been reluctant to switch from a fattening to a growth phase unless an excess of certain nutrients became available. Since the levels of carbon and nitrogen are believed to control fattening in other cases (15), this may also control the change here.

In summary, additions of metal salts had a slight stimulatory effect on growth rate. Since metal ions are often required as cofactors of enzymes, it seems reasonable that metal limitation would also influence growth rates. The magnesium content of milk is 120 mg/L, while manganese and iron are 22 and 450 µg/L, respectively (86). An optimum medium developed in chemostat cultures of baker's yeast contained 620 mg Mg (as 5.2 g of $MgCl_2 \cdot 6H_2O$), 50 mg Fe (as 0.35 g $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$) and 13.8 mg Mn (as $MnSO_4 \cdot H_2O$) per 10 L medium (62). The Mg content of milk would appear to be adequate but may be lowered when the casein is removed or whey proteins precipitated during autoclaving of the medium. Obviously these four organisms have slightly different metal requirements. Since iron is a constituent of the cytochrome systems in yeast, and Mn is a cofactor in fat synthesis, a requirement for these metals might be expected. None of the metal requirements are unusually high except, perhaps, the Mg requirement of 550 mg $MgSO_4$/10 L for T. cutaneum 40. This represents a

possible total of about 1800 mg Mg/10 L if all the Mg in milk remained in the whey. High metal ion concentrations have been associated with abnormal morphology in other yeasts (20) and were also observed in some cases with shake flask but not fermenter trials.

The addition of nitrogen stimulated the growth of all of these organisms and it must be concluded that nitrogen is the limiting nutrient in whey. Whey contains about 0.9% nitrogenous matter of which 0.5% is heat-coagulable protein (86). When the whey was autoclaved, probably only 0.4% soluble protein was available. Apparently, this amount was inadequate for growth.

Whey Fermentations at Optimum Conditions

Fermentation trials near optimum physical (Table 5) and nutrient (Table 6) conditions for these four organisms indicated that the two C. curvata strains were the more successful (Figures 6 through 13 and Table 7). Both had shorter generation times, produced fat and used lactose more rapidly than did the T. cutaneum strains.

Of the two C. curvata strains, R had the higher population, rate of fat accumulation, and the shorter fermentation time (Figures 6 and 7). Strain D (Figures 8 and 9) produced the most fat and had the largest fat coefficient (g fat/g lactose consumed x 100). These results illustrate the relation between cell numbers and fat yields. From the same amount of substrate, higher numbers of cells produce less fat than do lower cell populations, but lower cell populations generally increase fermentation times.

Table 6. Nutrient additions for optimum growth in whey for C. curvata strains R and D and T. cutaneum strains 24 and 40.

Additions/10 L	C. curvata		T. cutaneum	
	R	D	24	40
NH_4OH (15.5%)	10 ml	3 ml	10 ml	10 ml
$MnSO_4$	25 mg	-	20 mg	-
$MgSO_4$	-	-	30 mg	550 mg
$FeSO_4$	-	-	100 mg	-
K_2HPO_4	53 mg	-	-	-

Table 7. Summary of fermentation of whey under optimum conditions with C. curvata strains R and D and T. cutaneum strains 24 and 40.

	C. curvata		T. cutaneum	
	R	D	24	40
Generation time (hr)	1.31	1.05	1.88	1.60
Generations/hr	0.76	0.95	0.53	0.63
Maximum cell no. x 10^8/ml	6.5	5.2	6.5	3.4
Rate fat[a] produced	2.5	1.7	0.93	0.80
Rate lactose[b] used	32	32	38	17
Predicted[c] fat production	112	159	121	104
Predicted[c] lactose used	335	446	366	291
Soluble protein[d] start	55	44	51	41
Soluble protein[d] end	46	39	59	47
COD mg/L start	59,000	65,000	60,000	62,000
COD mg/L end	10,000	10,000	9,000	9,000
COD % reduction	71	85	85	85
Fat produced[d]	70	89	60	74
Fat coefficient[e]	16	19	13	16

[a]Maximum rate (g/hr/10 L) of production usually at <10 hr.

[b]Maximum rate (mM/hr/10 L) of utilization during the fattening period.

[c]Predicted from mass balance calculations and expressed as g/10 L.

[d]Calculated as g/10 L medium.

[e]Fat coefficient = g fat produced/g lactose used x 100.

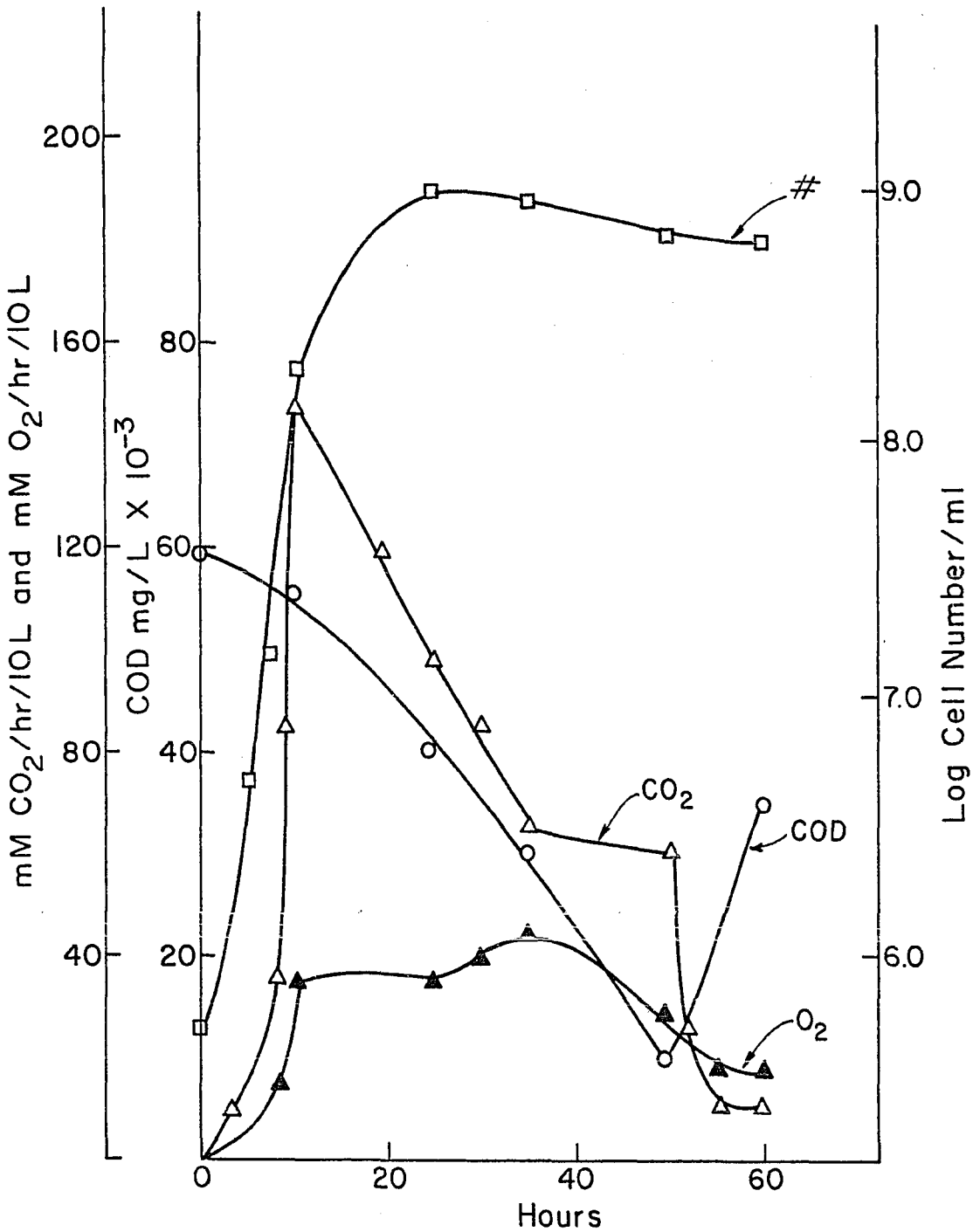


Figure 6. Respiration rate, cell numbers and COD during the fermentation of whey by C. curvata R. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

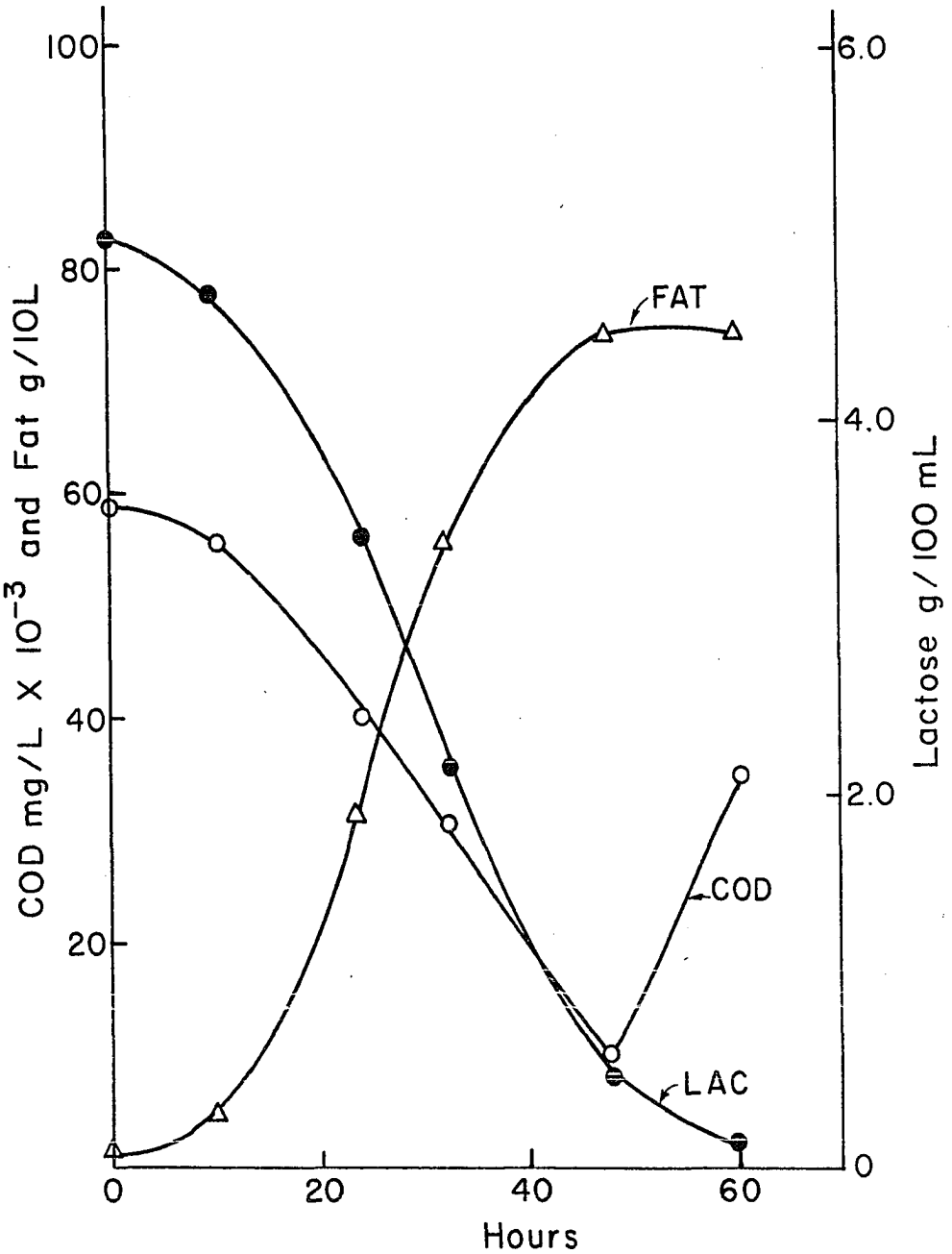


Figure 7. Lactose concentration, COD, and weight of fat during fermentation of whey by C. curvata R. ●Lactose, ○COD, △fat.

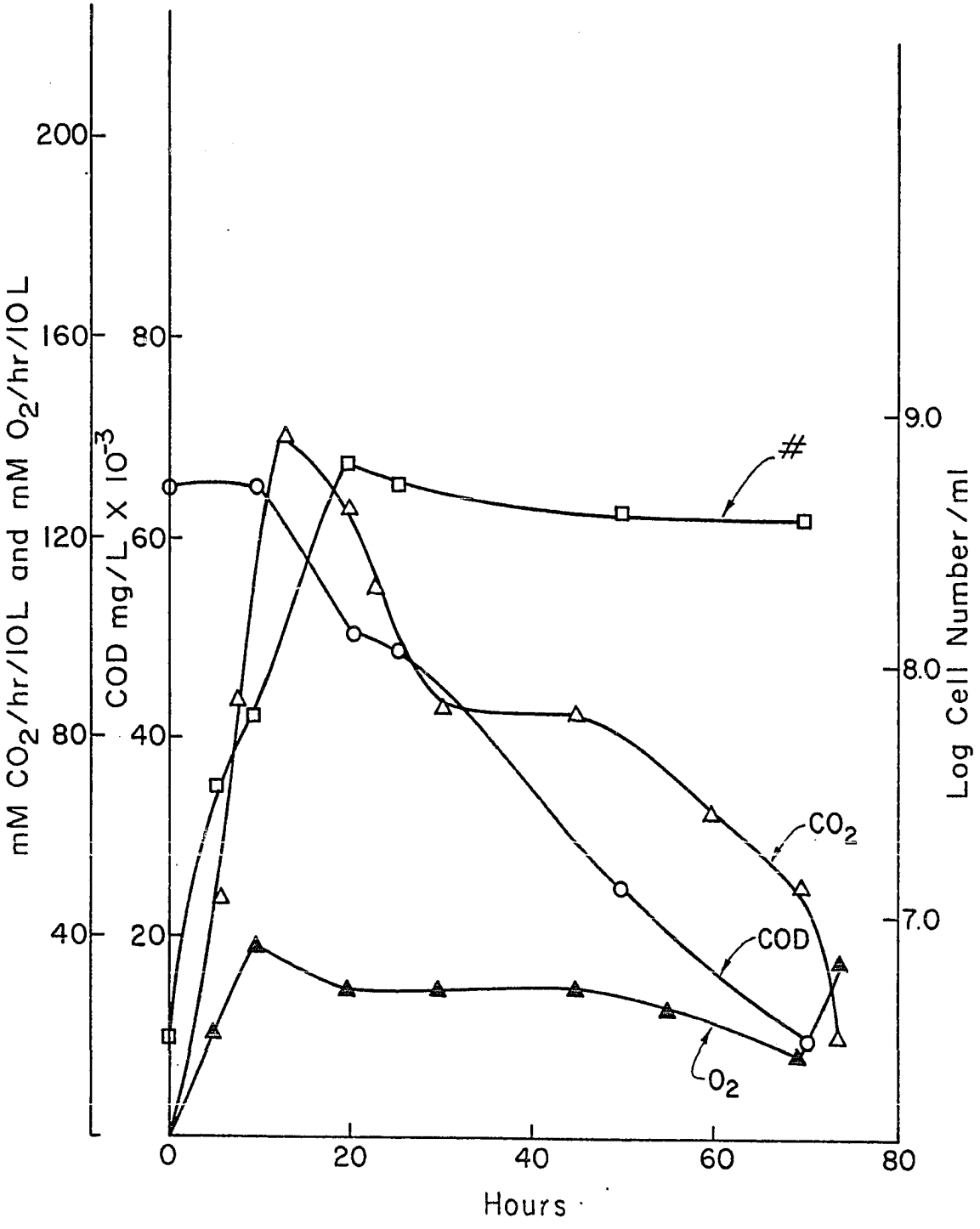


Figure 8. Respiration rate, cell numbers and COD during the fermentation of whey by C. curvata D. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

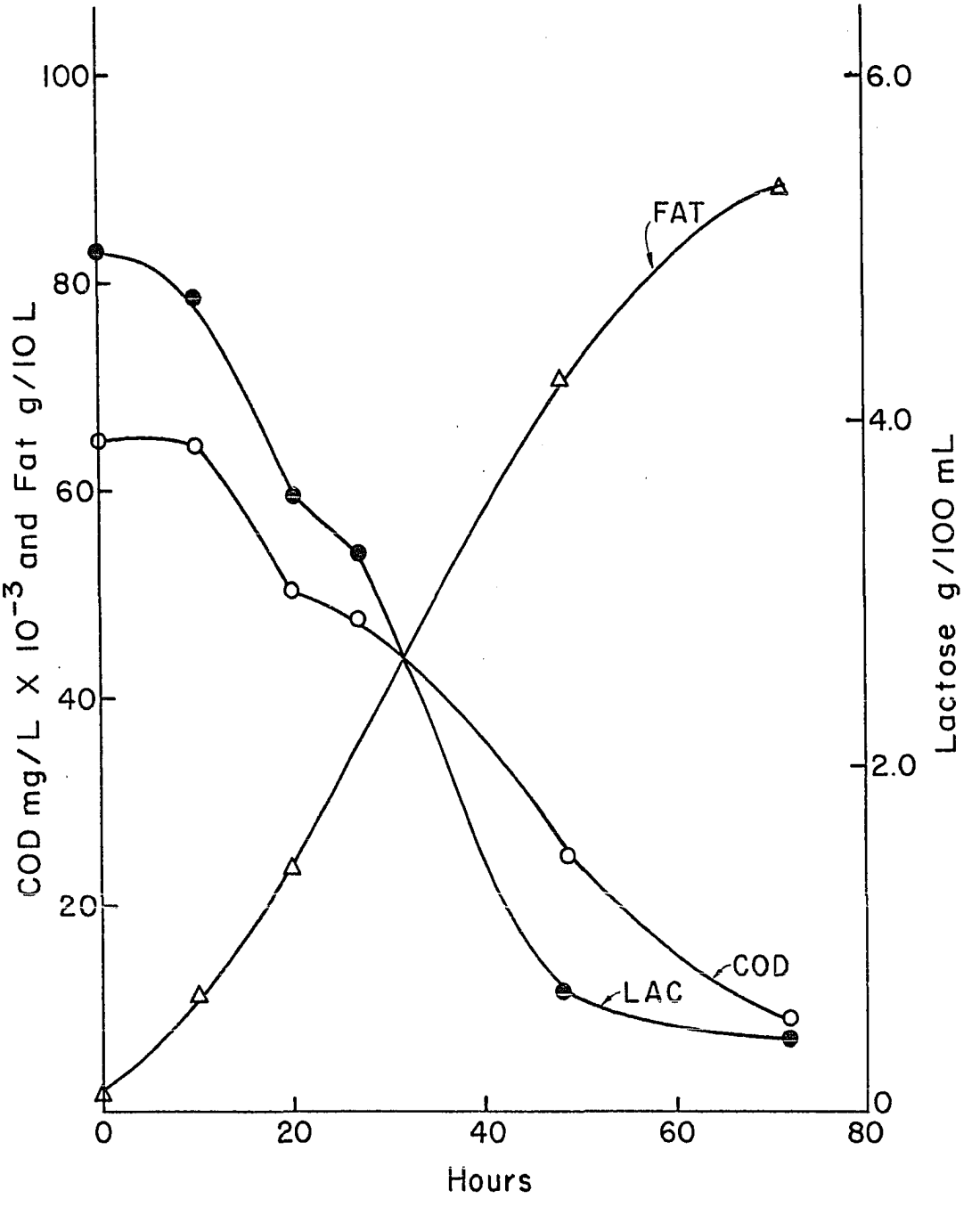


Figure 9. Lactose concentration, COD, and weight of fat during fermentation of whey by C. curvata D. ●Lactose, ○COD, △fat.

The relationship between cell numbers and fat production also was demonstrated by the two T. cutaneum strains (Figures 10, 11, 12, and 13). T. cutaneum 40 produced fewer cells but more fat. Both of these strains degraded some of the precipitated whey proteins, released soluble nitrogen into the medium and, presumably, used this nitrogen for growth.

The fat coefficients ranged from 13 to 19. If it is assumed that tripalmitin is the fat produced, a theoretical maximum coefficient of 38 can be calculated from mass balances.

In all cases the predicted fat production was greater than that actually recovered by about 50 to 70%. The predicted lactose used also underestimates the amount of lactose actually used. This is expected as the calculations do not allow for cell mass production. Free galactose percentages were low throughout the fermentations.

The residual COD was about the same in all fermentations. The lactose remaining at the end of the fermentation would account for a substantial portion of the terminal COD only in the case of C. curvata D. The other organisms reduced the original lactose by about 98%. The soluble protein accounted for roughly 50% (∿10,000 mg/L COD/g organic material) of the residual COD.

Fermentation of whey by all of these organisms was relatively inefficient, producing low yields of fat and leaving a substantial residual COD. If the precipitated protein removed with the cells is counted as residual COD, the total is probably about 15,000 mg/L. Since limits for sewage discharge from processing plants should be about 200 mg/L, this is an unreasonably high effluent COD. However, other attempts at COD reduction

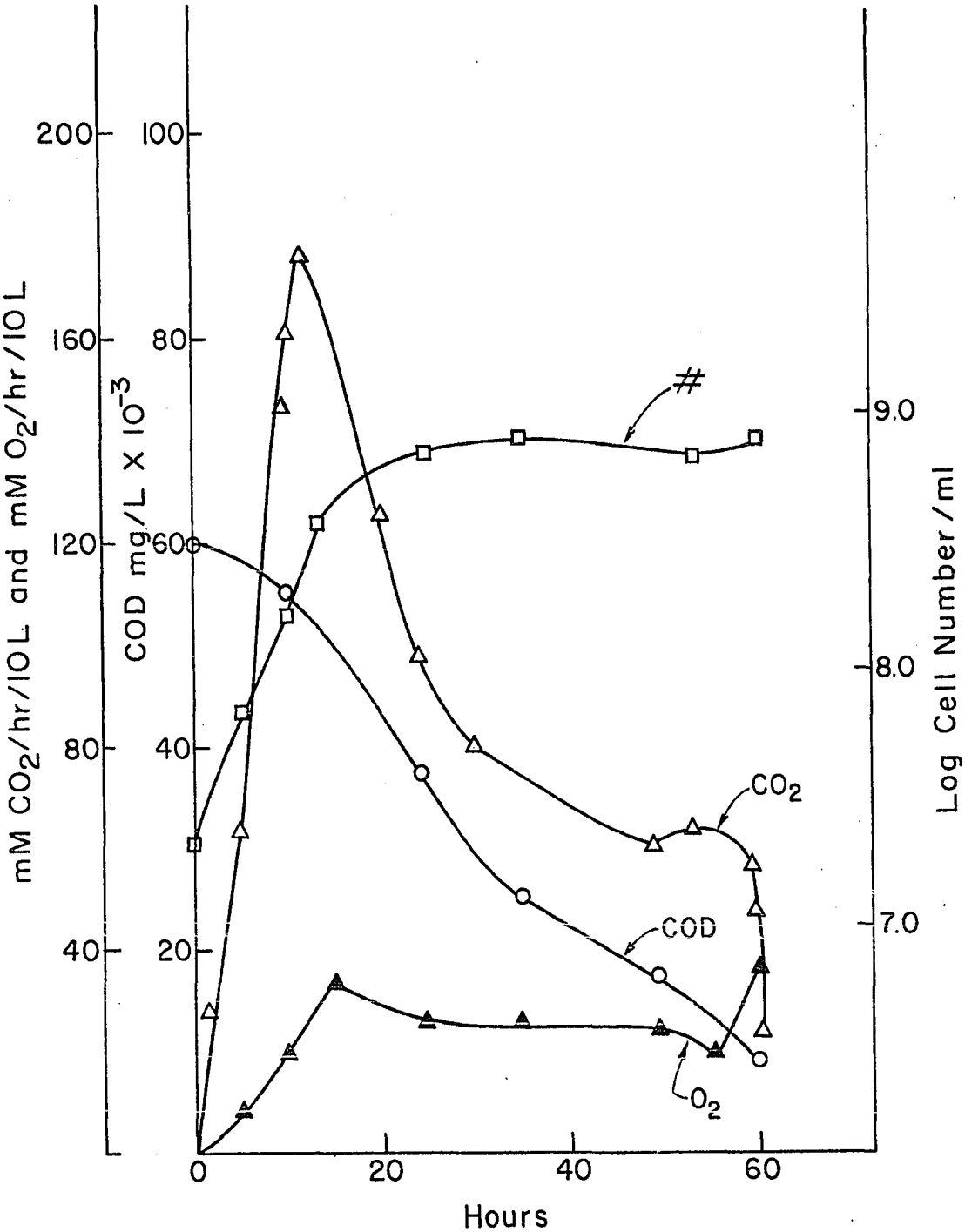


Figure 10. Respiration rate, cell numbers and COD during the fermentation of whey by T. cutaneum 24. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

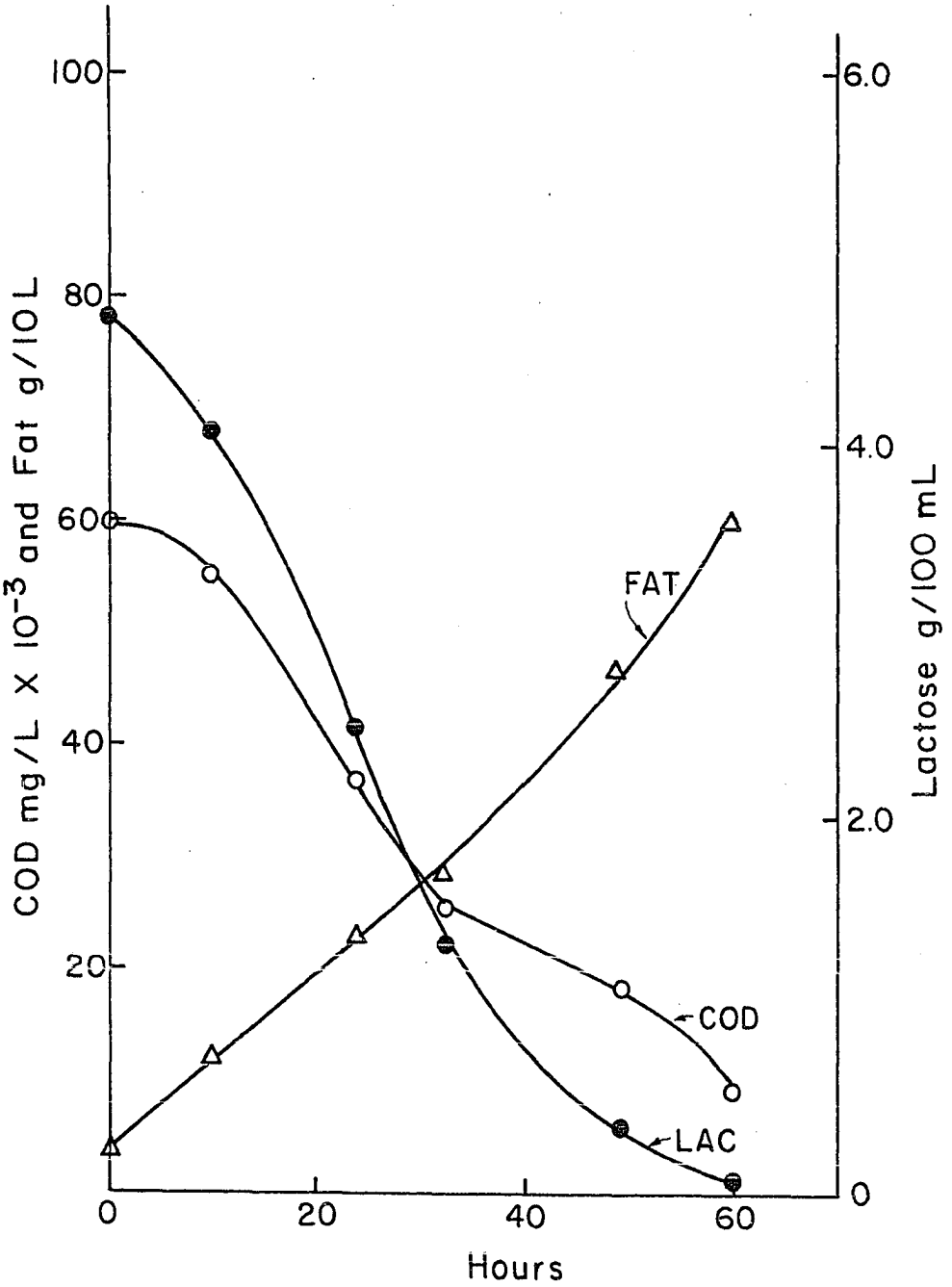


Figure 11. Lactose concentration, COD, and weight of fat during fermentation of whey by T. cutaneum 24. ●Lactose, ○COD, △ fat.

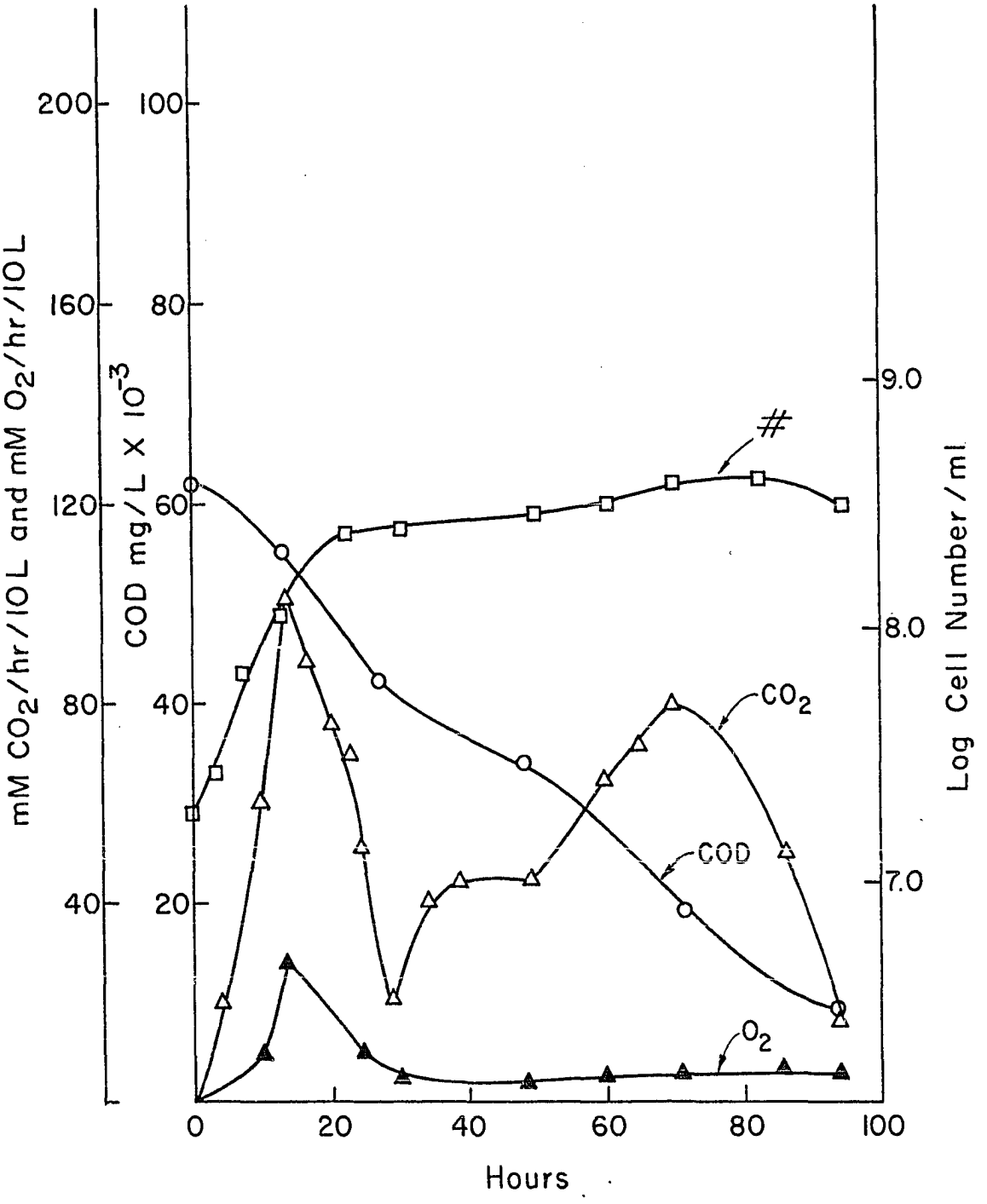


Figure 12. Respiration rate, cell numbers and COD during the fermentation of whey by T. cutaneum 40. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

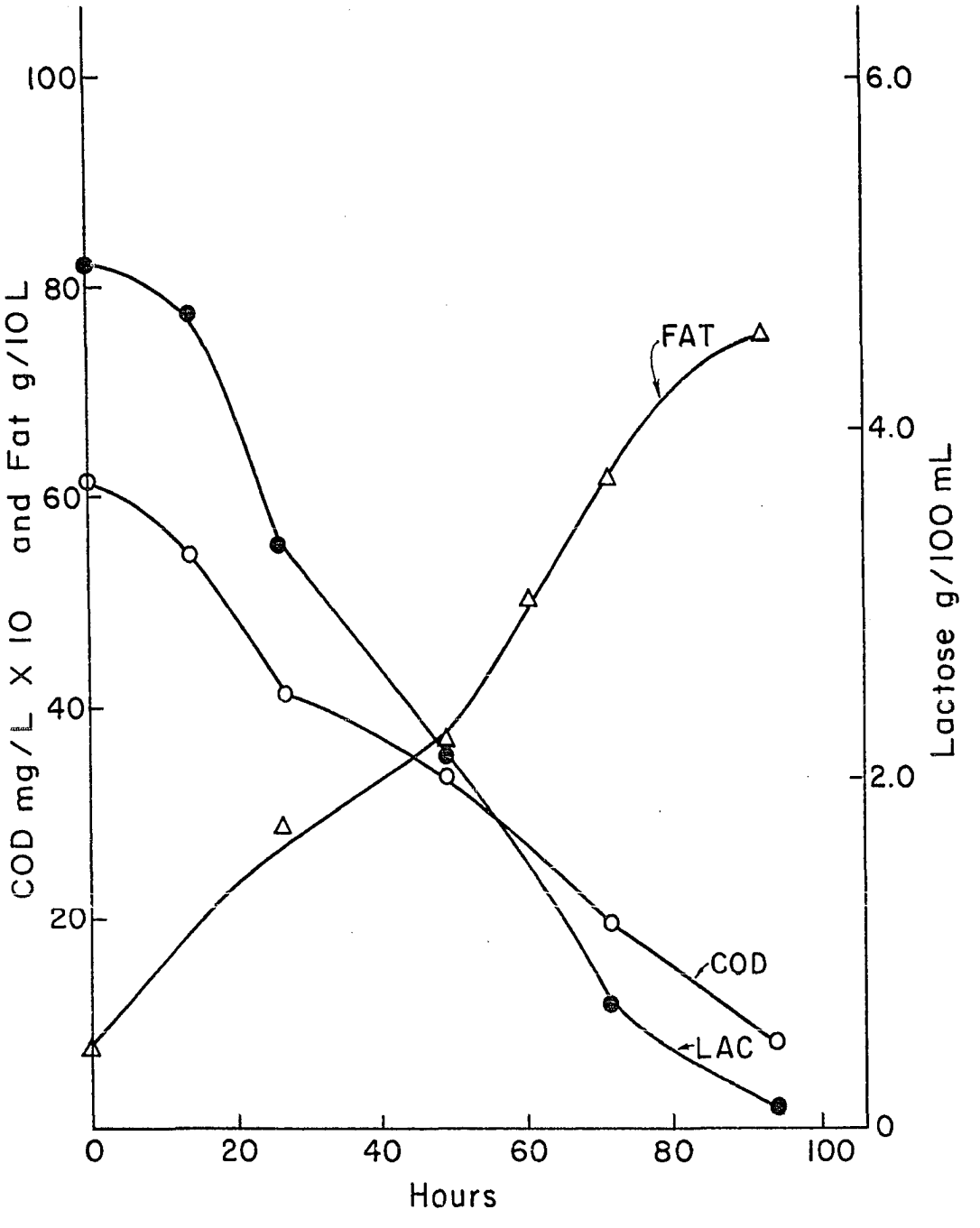


Figure 13. Lactose concentration, COD, and weight of fat during fermentation of whey by T. cutaneum 40. ● Lactose, ○ COD, △ fat.

of wastes also have been found to be about the same or less (72) successful.

It was possible that some inhibitory compound was present in the medium which prevented further COD reduction. When washed cells of T. cutaneum 24 were reinoculated into spent fermentation medium, growth occurred and was as rapid as in whey controls, but fat was not produced. In similar trials with C. curvata R, growth occurred but it was less than that observed with strain 24. Again fat was not produced. However, if lactose also was added to the spent medium fat production did occur. Further studies indicated that these two organisms can produce fat in lactose solutions without further nutrient additions. When washed cells harvested in stationary phase from whey-grown cultures were placed in 4% lactose solution, visual fattening occurred after 8 hr. Some lysis of cells had occurred after 8 hr and may have supplied some nutrients. Also, the cells may have been contaminated with whey constituents not removed during washing, but it seems likely that the nutrient requirements for fattening were not extensive. It was concluded that the organisms cease fat production because lactose is limiting.

The spent medium was examined to try and determine the composition of the residual COD. Since some yeasts produce excessive slime, fermenter supernatant was evaluated for nondializable COD. All of the organisms produced some slime (maximum about 0.5%). But, the slime was produced during logarithmic growth and seemingly was metabolized in the later phases of the fermentation. At the end of the fermentation, slime accounted for only about 500 mg/L COD. This was calculated by assuming

that of the 4,500 mg/L COD remaining after dialysis, about 4,000 was due to protein.

The residual COD seems to be mostly protein with small amounts of lactose and other components that are not easily utilized for fat production by these organisms.

Whey proteins are often removed from whey commercially by membrane ultrafiltration. Since these fat producers used the protein poorly, protein removal prior to fermentation would be advantageous economically. Fermentation of this filtrate, or permeate, also would have several distinct advantages for this study. Cell yields and composition could be much more accurately determined. In addition nitrogen balances could be more easily evaluated and cell protein production determined.

Whey Permeate Fermentations

The permeate was prepared to contain about 60-70,000 mg/L COD. Because all but 0.2% of the soluble nitrogen was removed in the filtration process, most of the COD was due to lactose. Required mineral nutrients and 10 to 11 ml of 15.5% NH_4OH were added to all fermentations.

Results are shown in Figures 14 through 21 and Table 8. Rapid cell growth and lactose utilization resulted in 72 hr fermentation times. This compares well with the fermentation of whey and indicates, as expected, that permeate is an adequate growth medium. Maximum cell populations for the four organisms were similar to those observed in whey except for C. curvata R which grew to 25% higher cell densities. The generation times for all organisms were slightly longer in permeate than in whey. This indicates that some nutrients were probably derived from whey proteins in

Table 8. Summary of fermentations of whey permeate under optimum conditions with C. curvata strains R and D and T. cutaneum strains 24 and 40.

	C. curvata		T. cutaneum	
	R	D	24	40
Generation time (hr)	1.63	1.10	1.89	2.0
Generations/hr	0.61	0.91	0.53	0.50
Maximum cell no. x 10^8/ml	8.7	5.2	6.5	3.7
Rate fat[a] produced	2.67	2.74	0.61	3.07
Rate lactose[b] used	28	33	15	24
Predicted[c] fat production	176	242	195	147
Predicted[c] lactose used	520	616	537	369
Cell mass[a] production	4.70	4.04	4.96	4.84
Lactose[d] start	650	574	578	471
Lactose[d] used	639	568	453	459
Protein[d,e] start	29.09	27.75	27.88	28.67
Protein[d,e] used	6.25	<.15	<.15	4.85
COD mg/L start	73,000	63,000	69,000	59,000
COD mg/L end	7,000	3,000	33,000	6,800
COD % reduction	90%	95%	52%	88%
Final cell yield[d]	257	268	196	239
Fat produced[d]	124	149	39	103
Cell protein[d]	22.54	24.71	29.18	32.19
Fat coefficient[f]	19	26	10	22

[a]Maximum rate (g/hr/10 L) of production usually at 40 hr.

[b]Maximum rate (mM/hr/10 L) of utilization during the fattening period.

[c]Predicted from mass balance calculations and expressed as g/10 L.

[d]Calculated as g/10 L medium.

[e]This includes the nitrogen added as NH_4OH and calculated as protein (%N x 6.25 = % protein).

[f]Fat coefficient = g fat produced/g lactose used x 100.

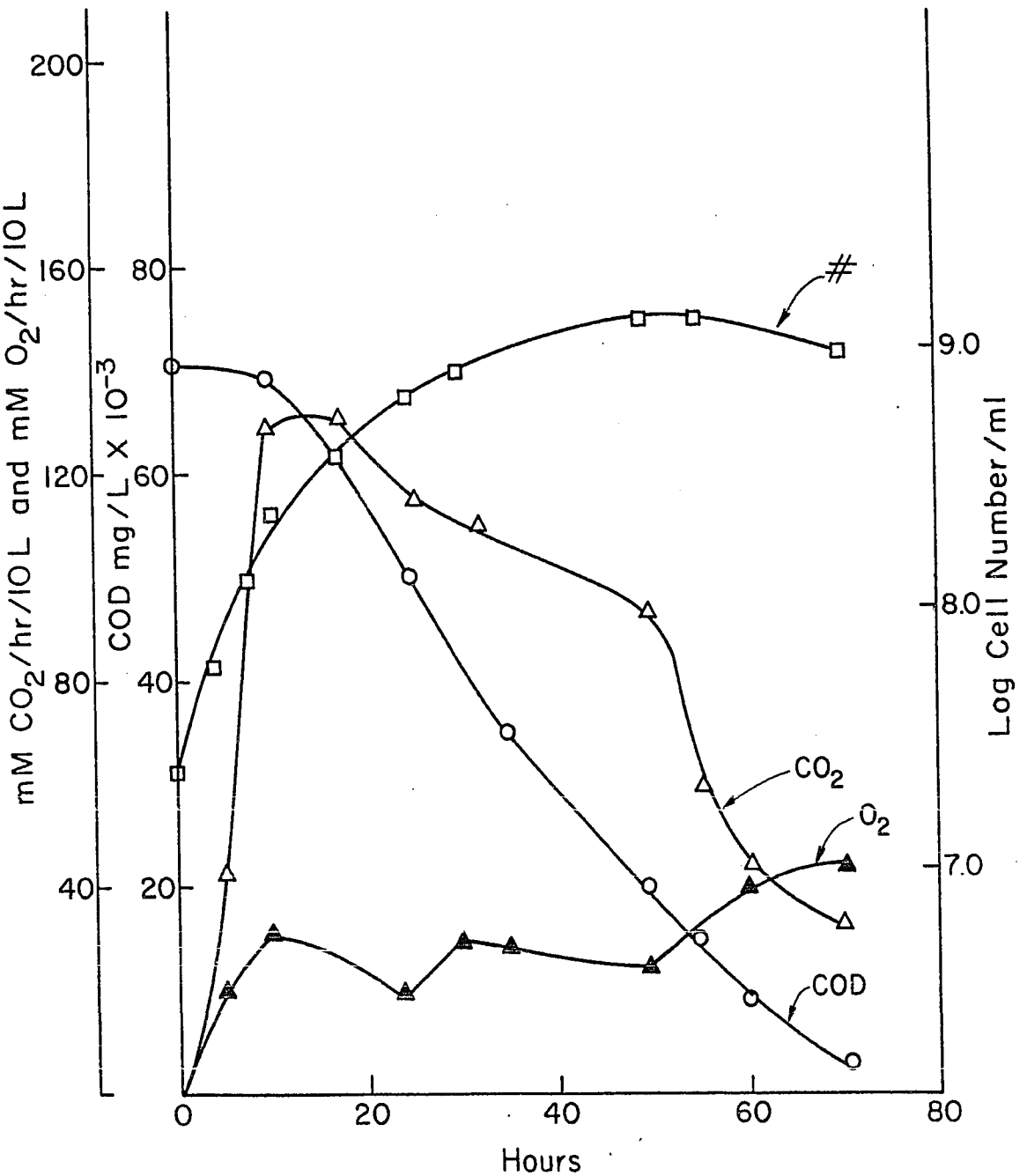


Figure 14. Respiration rate, cell numbers and COD during the fermentation of whey permeate by C. curvata R. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

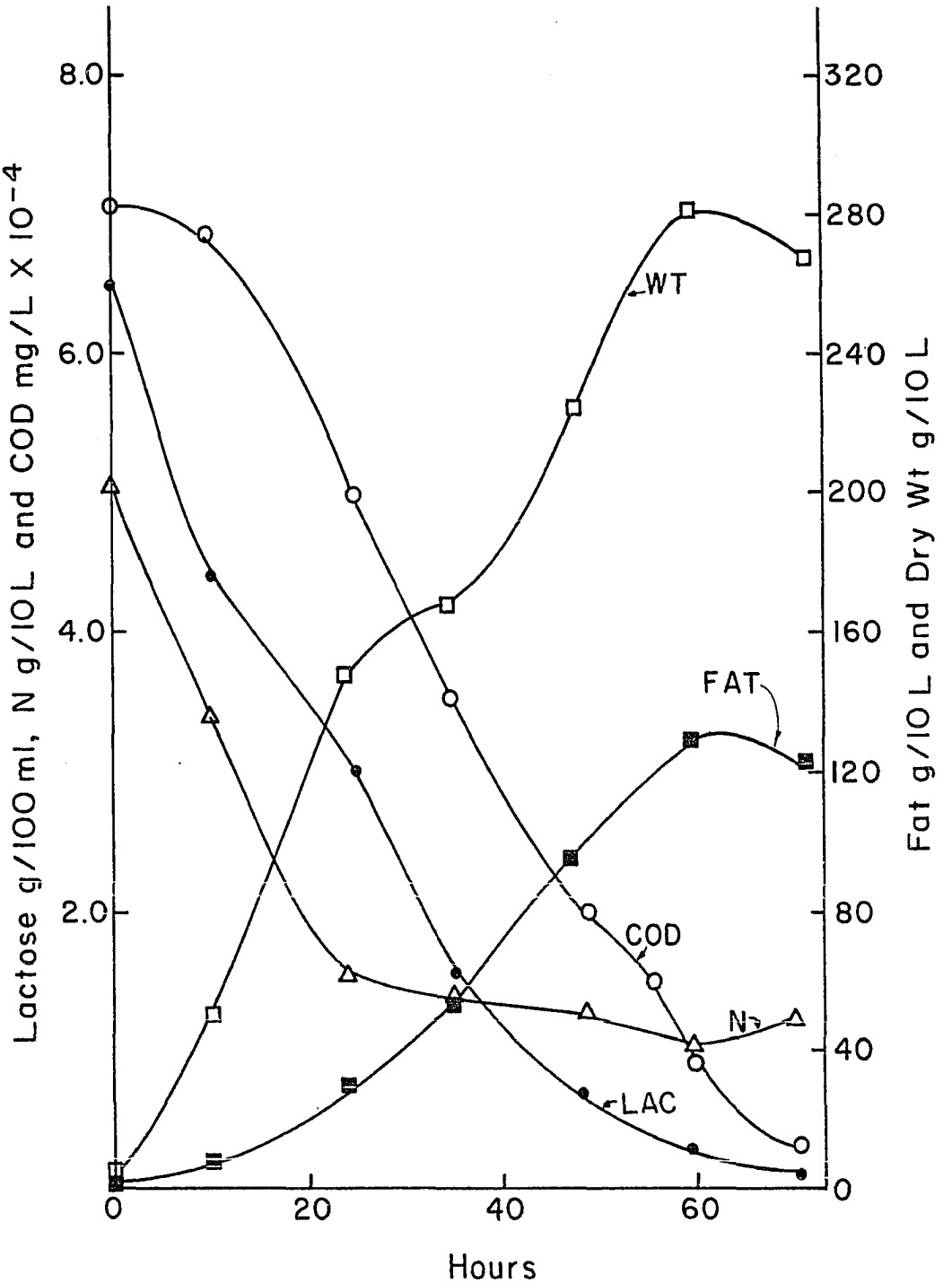


Figure 15. Lactose concentration, COD, weight of fat, cell weight, and weight of soluble nitrogen during fermentation of whey permeate by C. curvata R. ●Lactose, ○COD, ■fat, □cell weight, △ nitrogen.

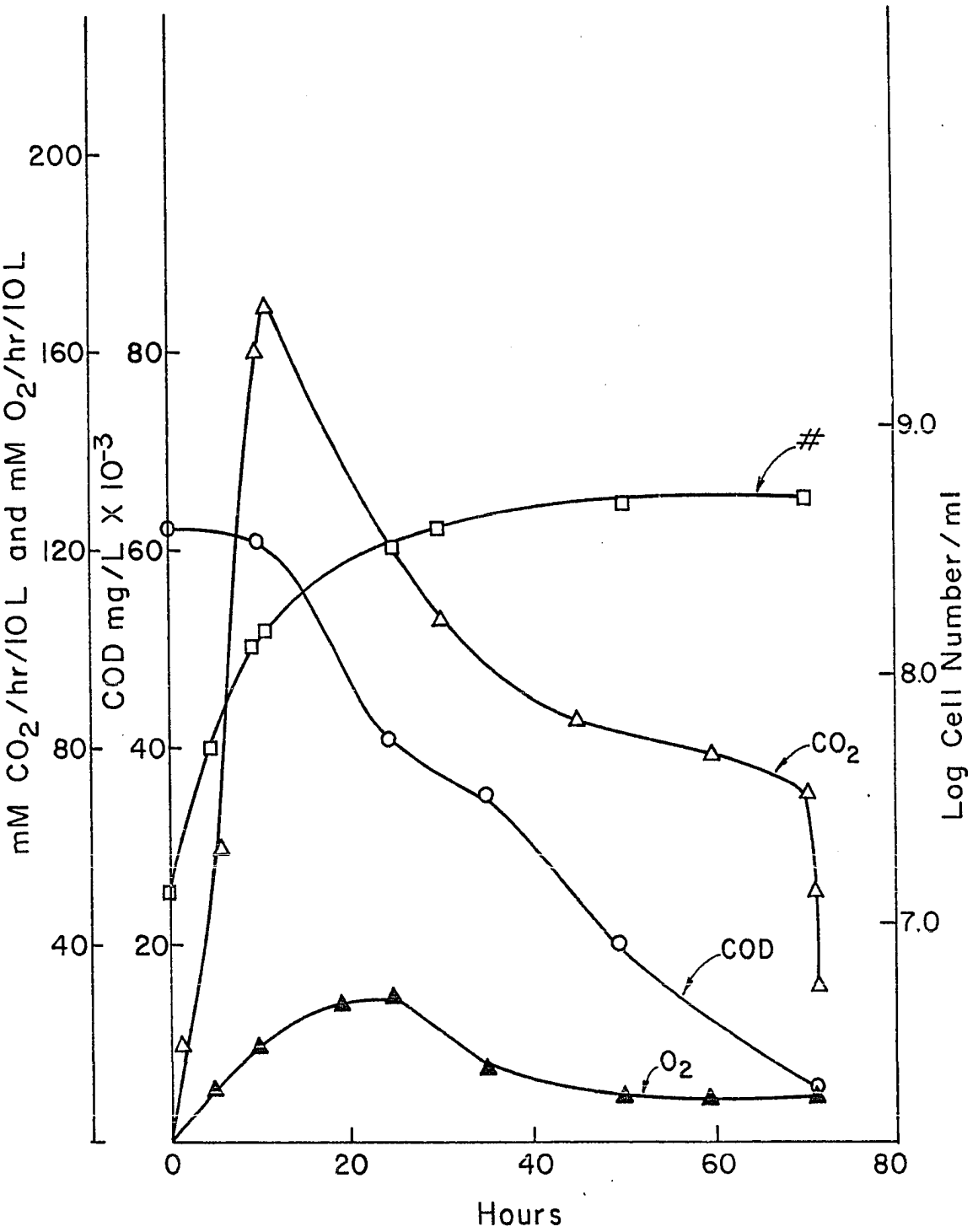


Figure 16. Respiration rate, cell numbers and COD during the fermentation of whey permeate by C. curvata D. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

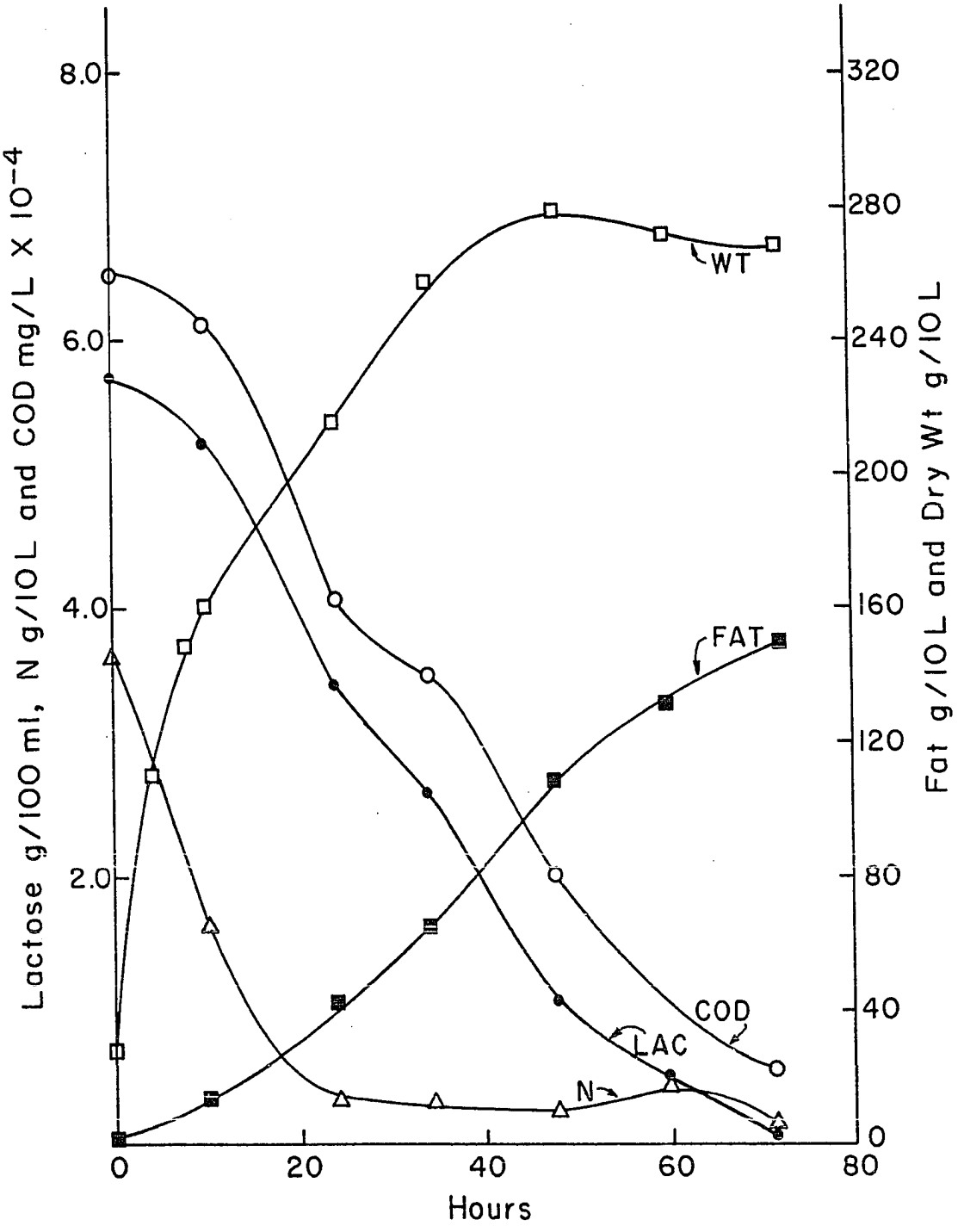


Figure 17. Lactose concentration, COD, weight of fat, cell weight, and weight of soluble nitrogen during fermentation of whey permeate by C. curvata D. ●Lactose, ○COD, ■fat, □cell weight, △ nitrogen.

earlier fermentations.

High rates of CO_2 production during logarithmic growth were observed in these permeate and whole whey studies. The maximum R.Q. was about 4.0 during the growth phase. If lactose is being used oxidatively, then the R.Q. should be more nearly 1.0. The high R.Q. may be due to fat production during this growth period, or the production of some partially oxidized organic end product. The presence of a distillable end product such as acetaldehyde or ethanol in the spent medium could not be demonstrated by gas chromatography. The pH rose during the early stages of growth, indicating that lactic acid was used, rather than that an acid product was produced. Utilization of lactic acid for growth would result in an R.Q. of 1.5 and would partially explain the high R.Q. Since the production of fat is continuous for these organisms (Table 10), this may also partially explain the high initial R.Q. and CO_2 production. The O_2-utilization measurements may also have underestimated the true values.

The reduction of COD was fairly linear in permeate and whey after the first 10 hrs of fermentation. This indicated the smooth utilization of lactose and no major production of extracellular COD. The slopes of the COD reduction and lactose utilization were similar, as is to be expected, in both the permeate and whey studies. The residual COD at the end of the fermentation is due to lactose, and extracellular slime which accounted for about 1000 mg/L of the residual COD.

The nitrogen was used rapidly during growth and after about 24 hr reached minimum values in most cases. This minimum coincided with the end of cell growth except possibly for strain R, which continued to divide

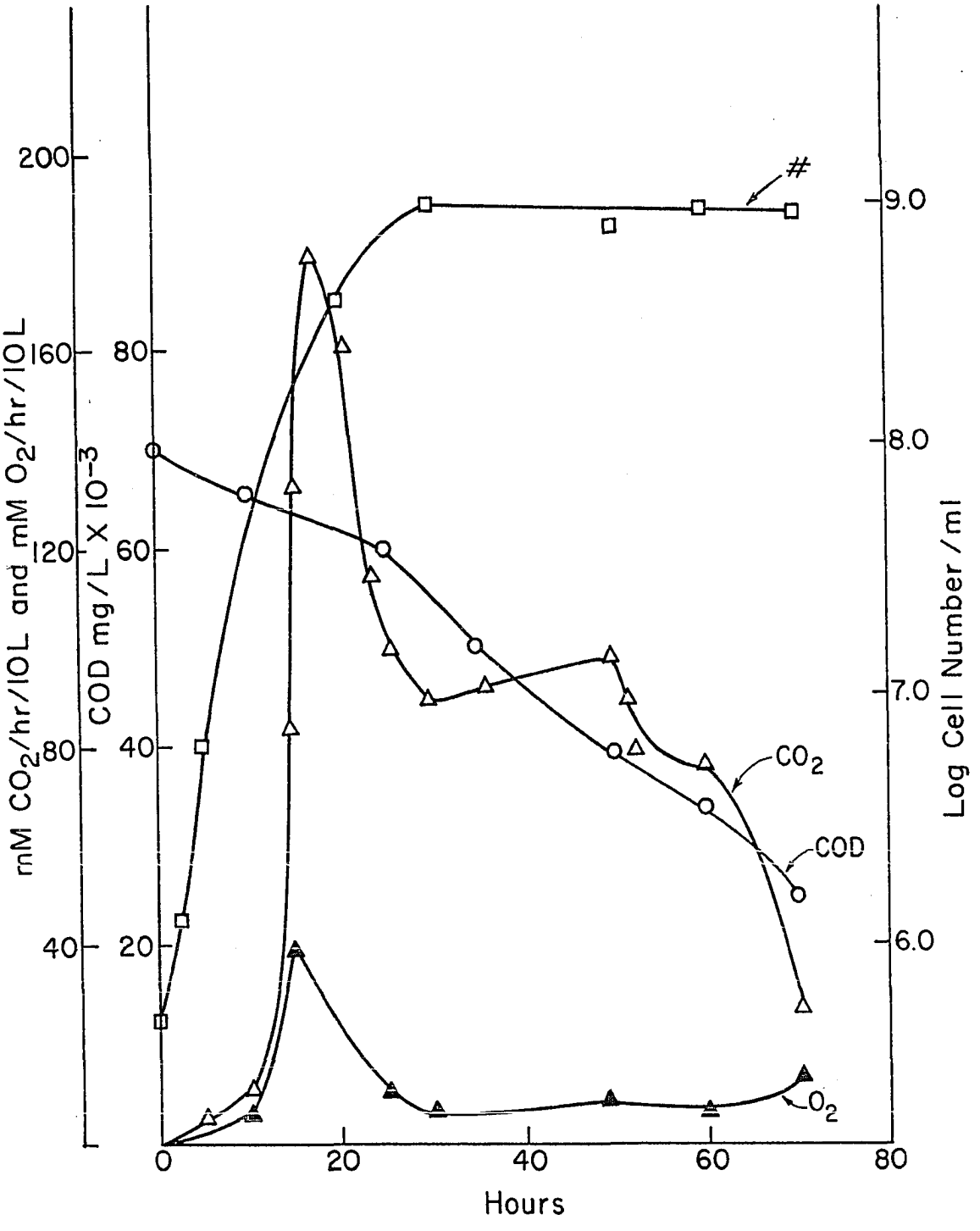


Figure 18. Respiration rate, cell numbers and COD during the fermentation of whey permeate by T. cutaneum 24. □ Log cell number, ○ COD, △ CO_2, ▲ O_2.

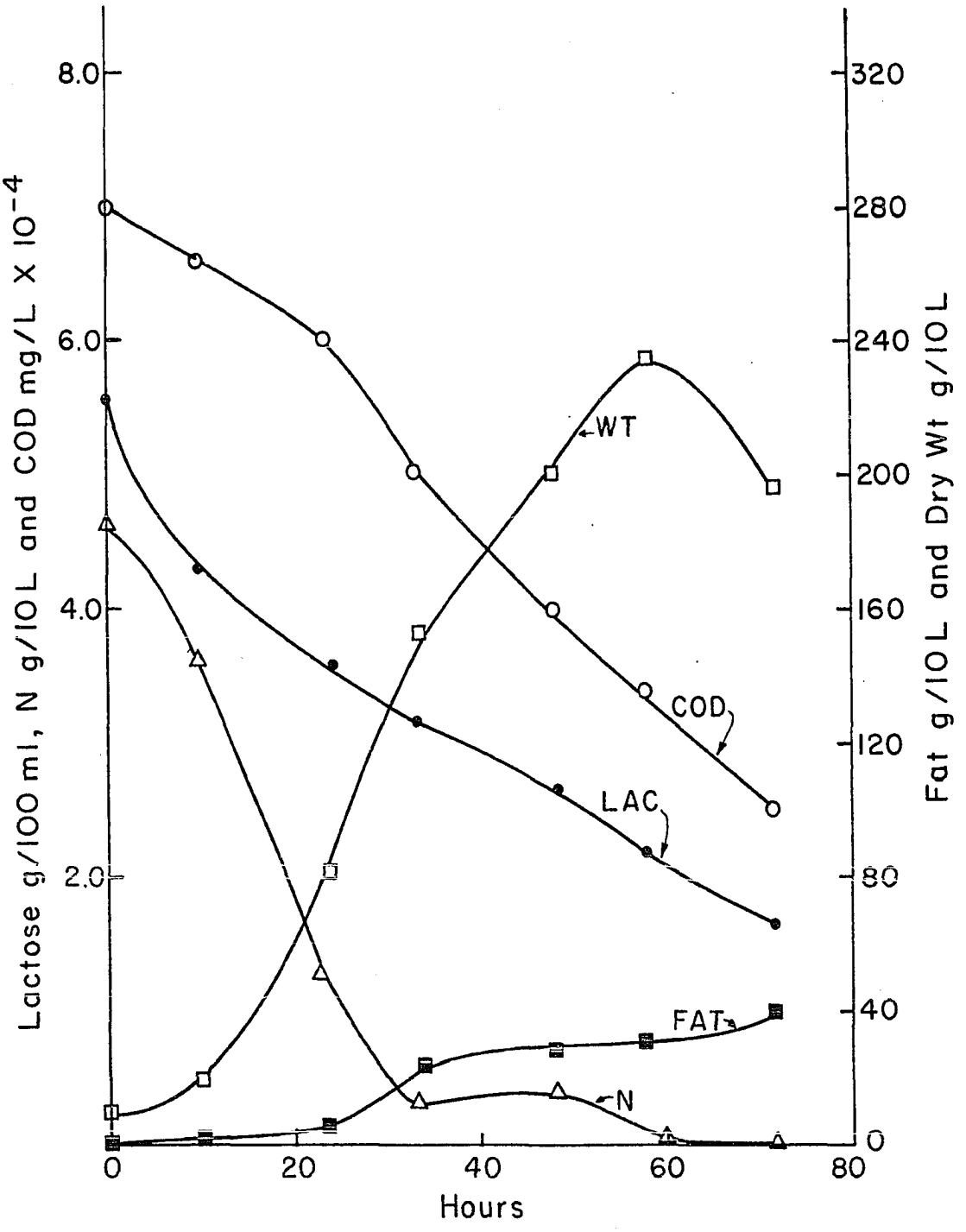


Figure 19. Lactose concentration, COD, weight of fat, cell weight, and weight of soluble nitrogen during fermentation of whey permeate by T. cutaneum 24. ● Lactose, O COD, ■ fat, □ cell weight, △ nitrogen.

slowly and utilize the residual nitrogen. Apparently, another nutrient besides nitrogen was growth limiting in this instance. The rapid accumulation of fat began very near the time nitrogen was exhausted and growth ceased. This was the expected result and was demonstrated in permeate more readily than in the whey experiments where nitrogen measurements could not be made.

Cell mass accumulation proceeded smoothly until near the end of the fermentation period; then cell weight/ml declined for all organisms except strain 40. This decline was probably due to cell lysis; however, the R.Q. for R was close to 1.0 after 60 hr indicating that fat was used for energy. The production of fat paralleled the cell mass increase in the later stages of fermentation. However, the accumulation of fat didn't account for the total increase in cell mass since only about 66% of the increase in cell mass was recovered as fat. The organisms must have been accumulating other unidentified cell material.

The fat production rate for T. cutaneum 24 was considerably lower than for the other organisms. Lactose was also used more slowly and final COD reduction was very poor. These results are in contrast to the more rapid and complete fermentation in whey by this organism. The poor fermentation of permeate is probably due to some nutrient deficiency that was previously supplied by the whey proteins. It had been observed earlier that this strain may have preferred preformed amino acids. It is also possible that some mineral component of the whey that was essential for fattening was removed during filtration.

The other three organisms were much more successful in permeate and

compared well with the whey fermentations. More fat was produced in these fermentations than in whey, and reached 56% of the dry weight of C. curvata D. This organism, as did strain 40, began to accumulate fat after about 10 hr and reached a yield of 149 g/10 L at 72 hr at a fat coefficient of 26. C. curvata D reached maximum cell mass at 48 hr but continued to produce fat until 72 hr. Perhaps some intracellular carbohydrate reserve was used in fat production. In C. curvata R the fat yield went down at 72 hr to 124 g. Lysis may have obscured the maximum fat yield, especially in C. curvata R. T. cutaneum 40 was surprisingly successful in permeate producing 103 g fat/10 L at a very rapid rate after 48 hr. The fat produced in whey was only 74 g/10 L and was produced at a much slower rate.

As expected the fat predicted from mass balance equations do not accurately predict the fat produced and lactose used because they do not provide for cell mass accumulation. Similar trends were observed in the whey fermentations. The CO_2 and O_2 measurements did serve as a useful tool to monitor the progress of the fermentations.

The final cell yields of 196 to 268 g/10 L are considerably higher than other reports of cell yields (3,54,63). The % fat dry wt. is about the same as in these studies but the total fat yield is higher due to increased cell yields. The fat coefficient for these studies is also slightly higher than previous reports (45,54,63) but is lower than that of 40 reported by Allen et al. (3) which is very near the theoretical maximum.

The protein content is 10 to 15% dry wt. depending on the organism. After the fat is removed by extraction the residual cell paste would be 17 to 25% protein. This is a fairly low protein content because most

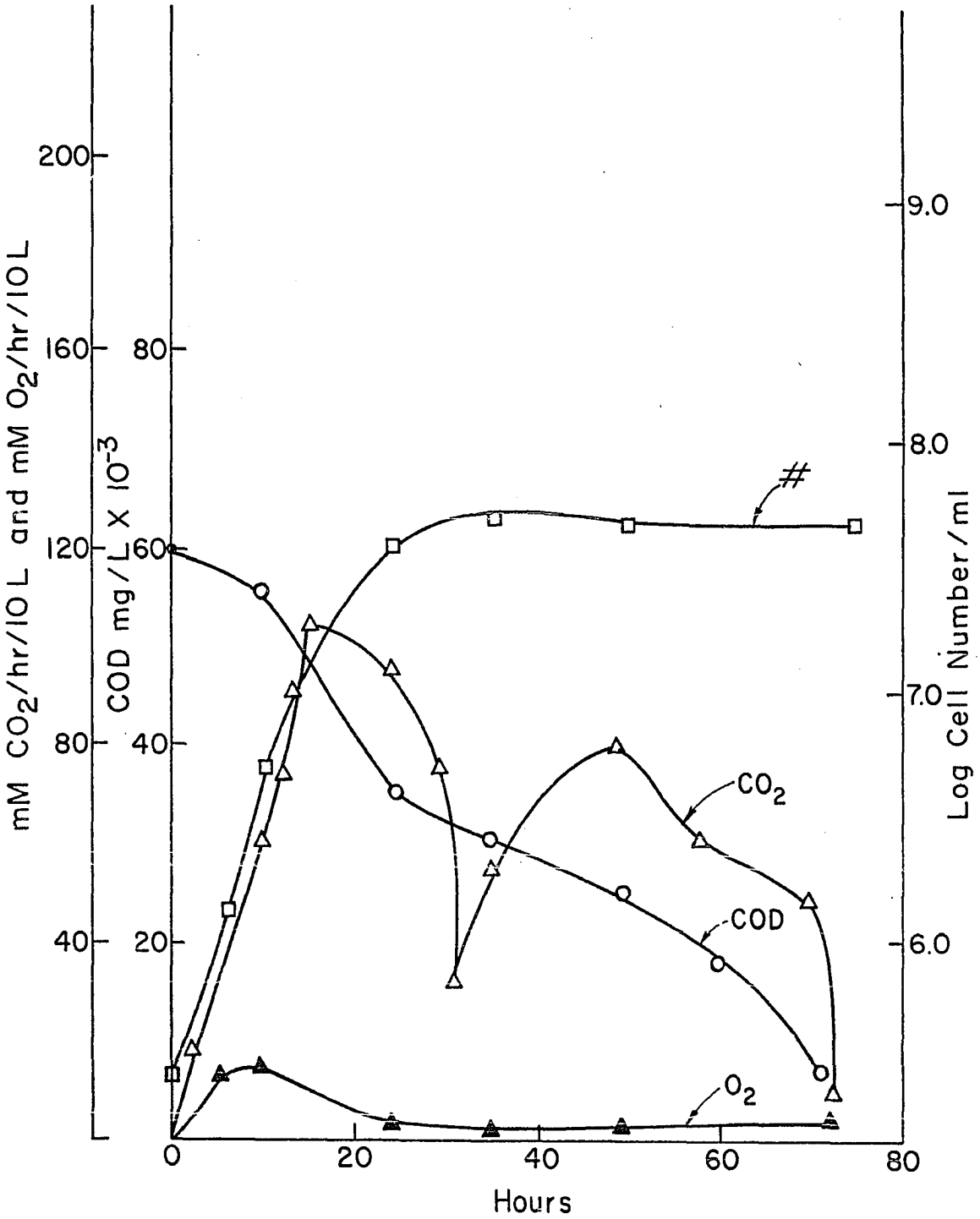


Figure 20. Respiration rate, cell numbers and COD during the fermentation of whey permeate by T. cutaneum 40. □Log cell number, ○COD, △CO_2, ▲O_2.

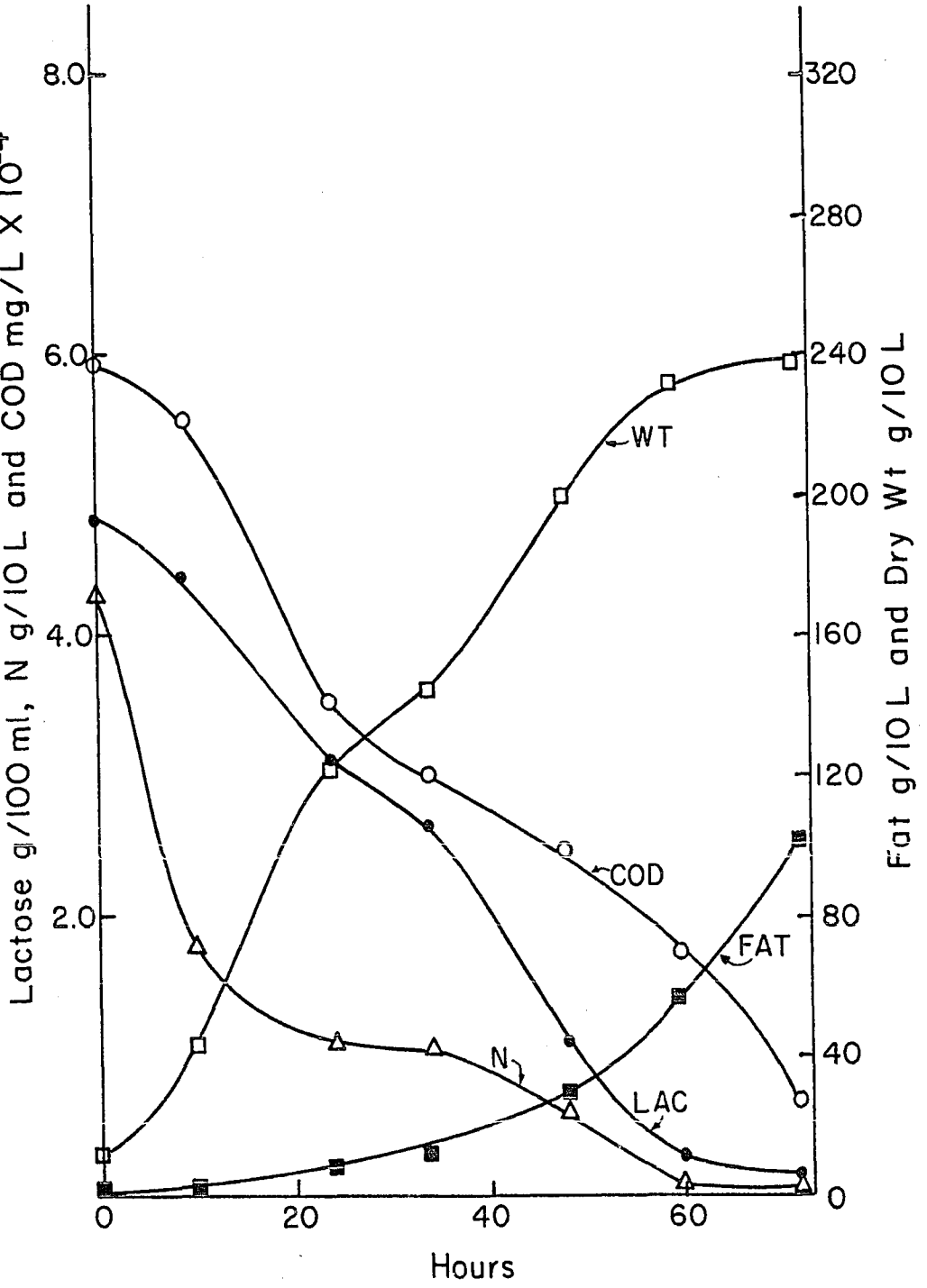


Figure 21. Lactose concentration, COD, weight of fat, cell weight, and weight of soluble nitrogen during fermentation of whey permeate by T. cutaneum 40. ● Lactose, ○ COD, ■ fat, □ cell weight, △ nitrogen.

cells such as S. fragilis contain about 40% protein (49).

In summary, C. curvata D was the most successful fermenter of whey and permeate. It produced the most fat, reduced the COD to low levels and required the fewest nutrient additions (only nitrogen was stimulatory). C. curvata R was also very successful and may have produced more fat and higher cell weights at 65 hr before lysis began. T. cutaneum 24 was fairly successful in whey but not in permeate, presumably because of nutritional requirements. T. cutaneum 40 was more successful in permeate than in whey, producing about 100 g/10 L fat. The COD of permeate was reduced about 90-95% by the most successful organisms.

Lipids Produced

No single method for fat extraction from yeasts yields 100% recovery of all lipid classes (74), so the fat composition varies with the extraction method used. It would be desirable to extract all the lipids from intact cells nondestructively, but very low yields were obtained with solvent extractions, even after attempts to disrupt the cells mechanically. The KOH digestion method gave the highest recoveries. This method is destructive and recovers only fatty acids and nonsaponifiables, so it tends to underestimate the amount of lipid. The Folch et al. (28) method was used in the initial fermentation screening (Table 2) and probably severely underestimates the fat produced.

The lipid composition of C. curvata and T. cutaneum strains varied with the growth temperature, the age of the cells, and the composition of the medium (Table 9 and 10 and Figures 26 through 29).

Table 9. The fatty acid composition of two strains each of C. curvata and T. cutaneum in different media and at different temperatures.

Organism	Medium	% fatty acid				
		$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
C. curvata R	Whey	2	26	10	54	9
	Whey permeate	Tr[a]	31	12	51	6
	Whey 15C[b]	Tr	19	6	62	10
C. curvata D	Whey	1	25	10	57	7
	Whey permeate	Tr	32	15	44	8
	Whey 15C	Tr	22	6	67	5
T. cutaneum 24	Whey	3	13	22	50	13
	Whey permeate	Tr	21	33	29	10
	Whey 15C	Tr	20	11	59	10
T. cutaneum 40	Whey	2	30	13	46	10
	Whey permeate	Tr	30	13	46	11
	Whey 15C	Tr	25	22	51	2

[a]Tr = Detected in amounts less than 1% of total fatty acids.

[b]Whey agar medium incubated at 15C.

Table 10. The % fat of the dry weight of two strains each of C. curvata and T. cutaneum during fermentation of whey permeate.

Time hr	C. curvata		T. cutaneum	
	R	D	24	40
0	40	11	11	10
10	18	9	5	4
24	20	20	9	7
34	32	26	10	8
48	35	39	9	15
60	46	49	13	25
72	47	56	20	43

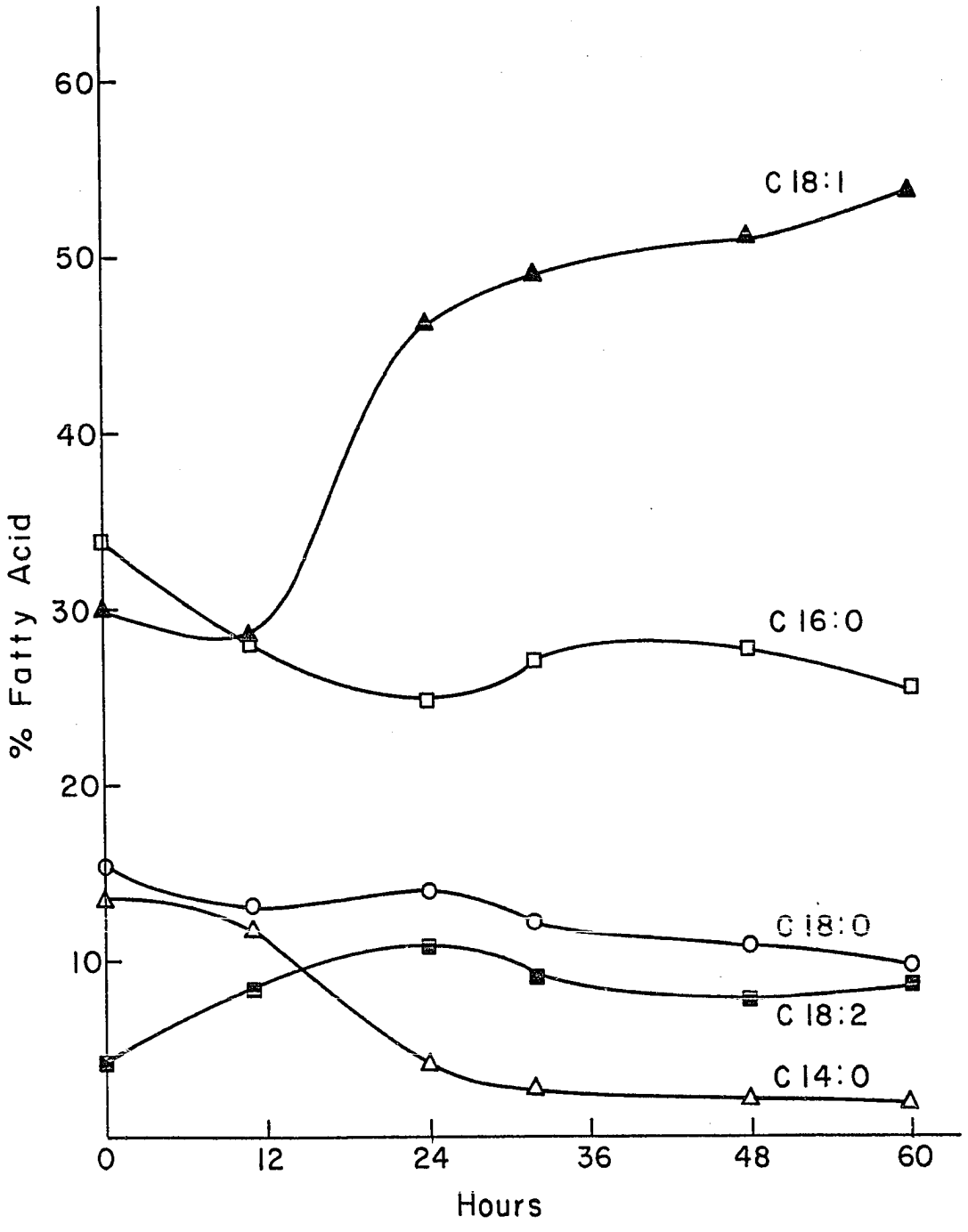


Figure 22. The change in fatty acid composition during the fermentation of whey by C. curvata R. △ 14:0, □16:0, ○18:0, ▲ 18:1, ■18:2.

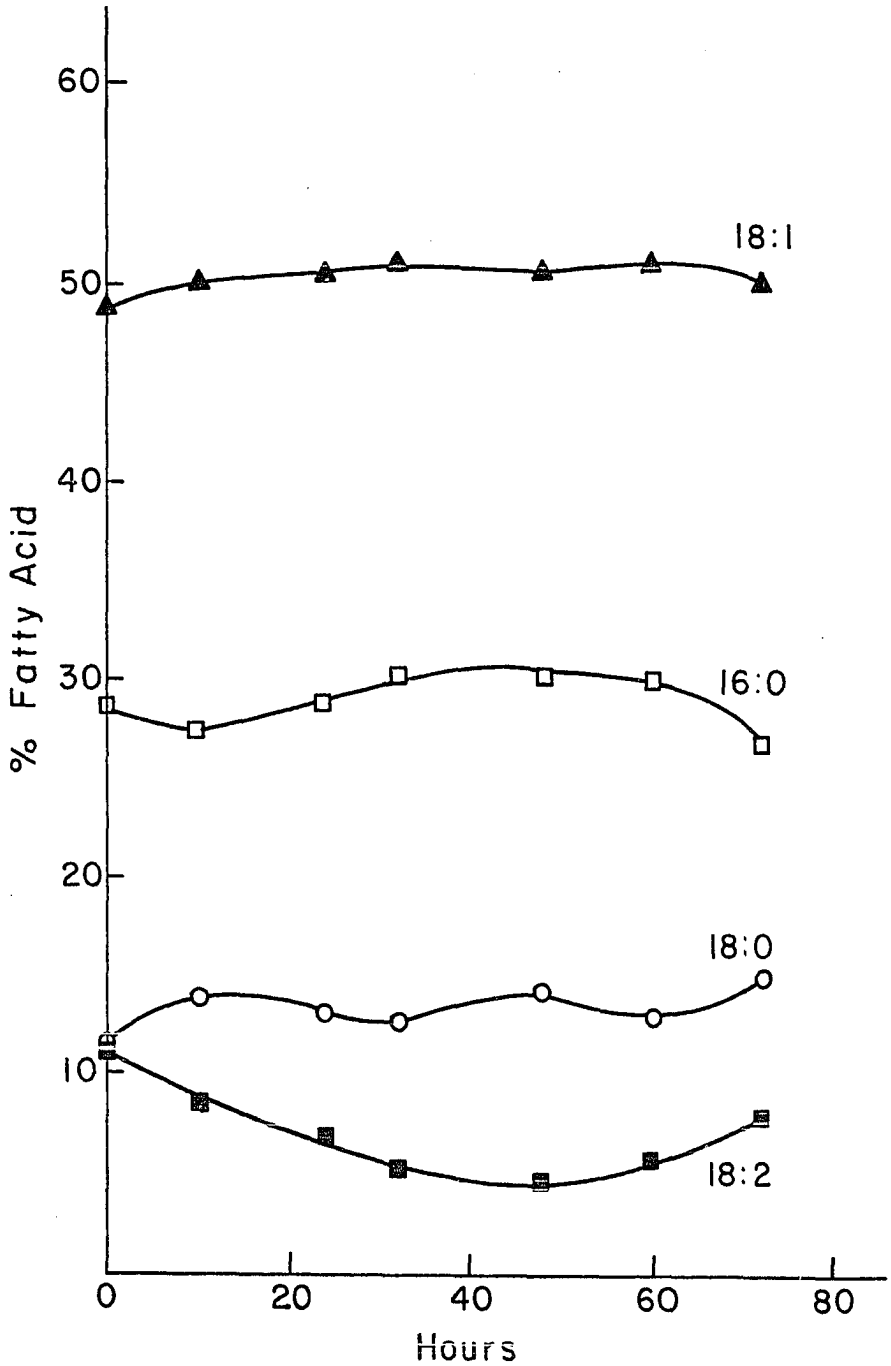


Figure 23. The change in fatty acid composition during the fermentation of whey permeate by C. curvata R. □16:0, ○18:0, ▲18:1, ■18:2.

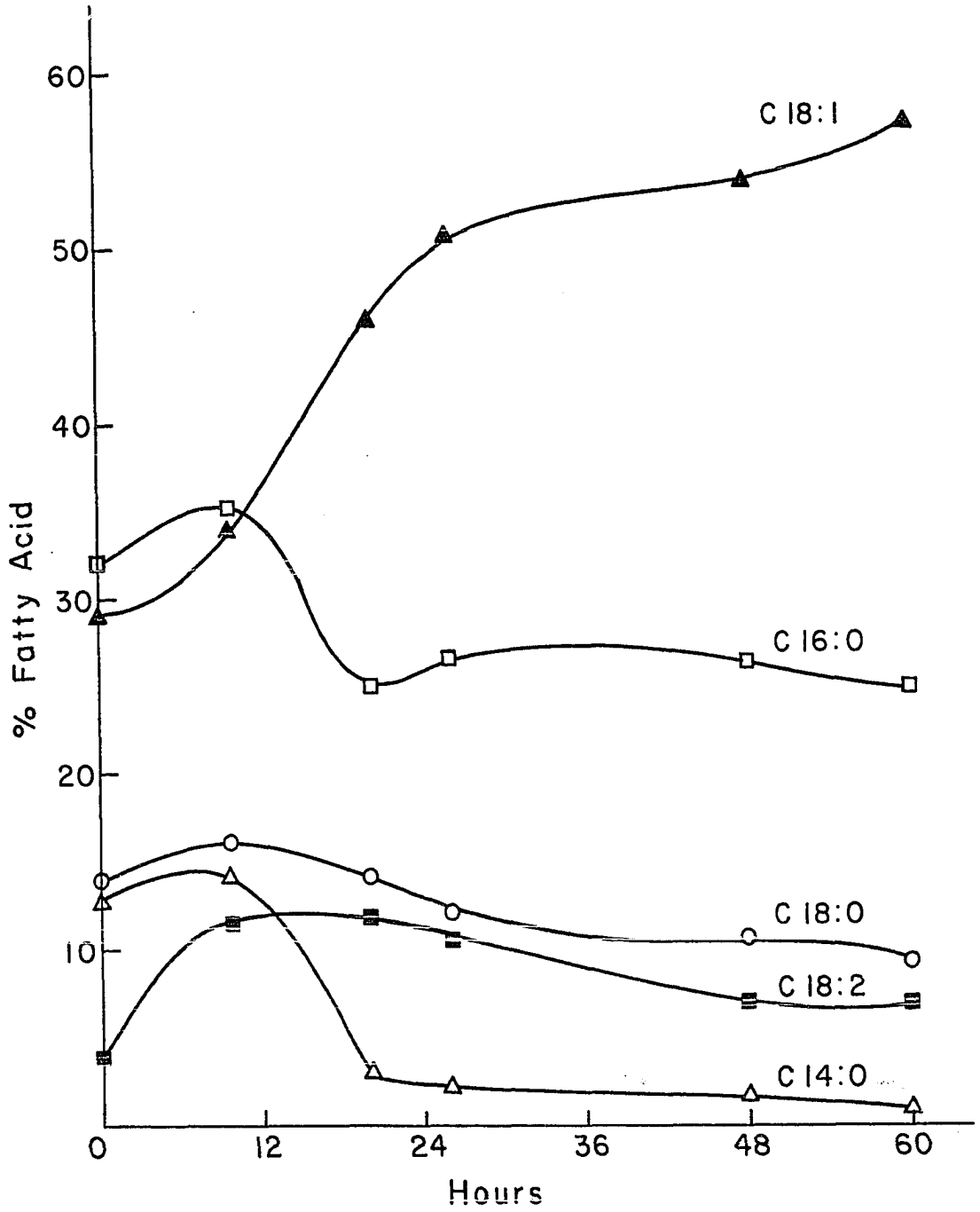


Figure 24. The change in fatty acid composition during the fermentation of whey by C. curvata D. △ 14:0, □ 16:0, ○ 18:0, ▲ 18:1, ■ 18:2.

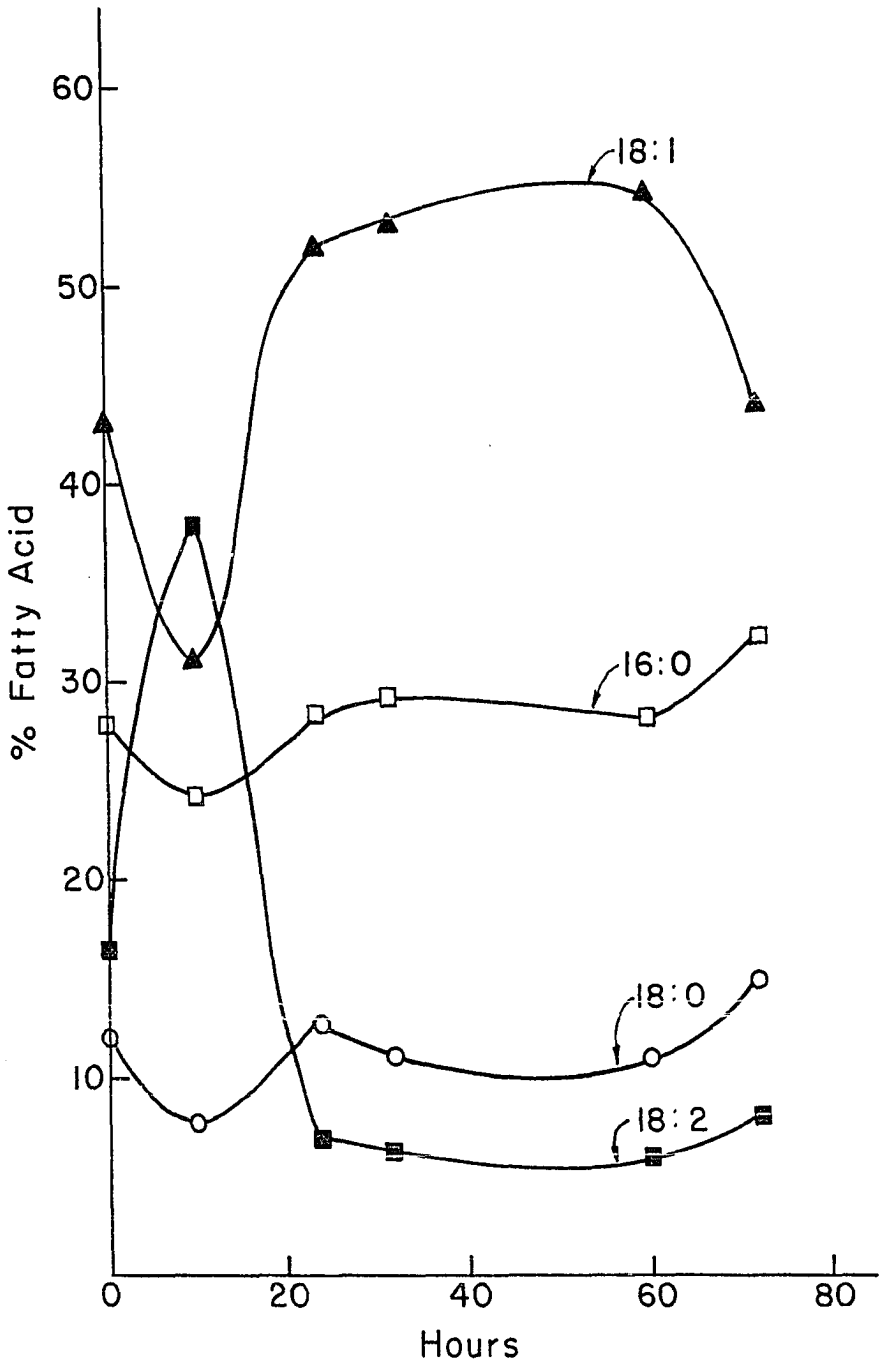


Figure 25. The change in fatty acid composition during the fermentation of whey permeate by C. curvata D. □16:0, ○18:0, ▲18:1, ■18:2.

Table 9 shows the lipid composition of these organisms when grown near their respective optimum growth temperatures in whey and permeate and at 15C in whey. In general, all four organisms are rich in oleic acid and also contain substantial amounts of palmitic and stearic acids. The fat is relatively low in polyunsaturated fats which would probably make it very stable to oxidation. Other yeasts such as R. gracilis and Debaromyces hansenii NRRL Y-1448 have been reported to have a similar lipid composition (87). The oil also has a fatty acid composition similar to pig fat and beef tallow although T. cutaneum 24, when grown in whey, more closely resembles cocoa butter (88). The physical properties of the fat depend on the glyceride structure as well as the fatty acid percentages. The actual physical characteristics, that help to determine its market value, may be quite different from lard and tallow.

When the growth temperature was lowered to 15C, more oleic and less stearic acids were produced. T. cutaneum 40, however, produced slightly more stearic acid. Other organisms also increase the degree of unsaturation of their lipids at decreased temperature to maintain partial lipid fluidity (21). The ratio of saturated to unsaturated fatty acids decreased from 1:1.66 to 1:2.88 at 15C.

It is possible to estimate the melting point of the fat by comparing its fatty acid composition to other fats. Tallow and lard completely melt at 40 to 48C. Since this is above their body temperature, it must be concluded that the fat is partially solid at body temperature. This appears to be true also with these organisms. However, sterols in the fat or other components can drastically alter the crystal structure of the

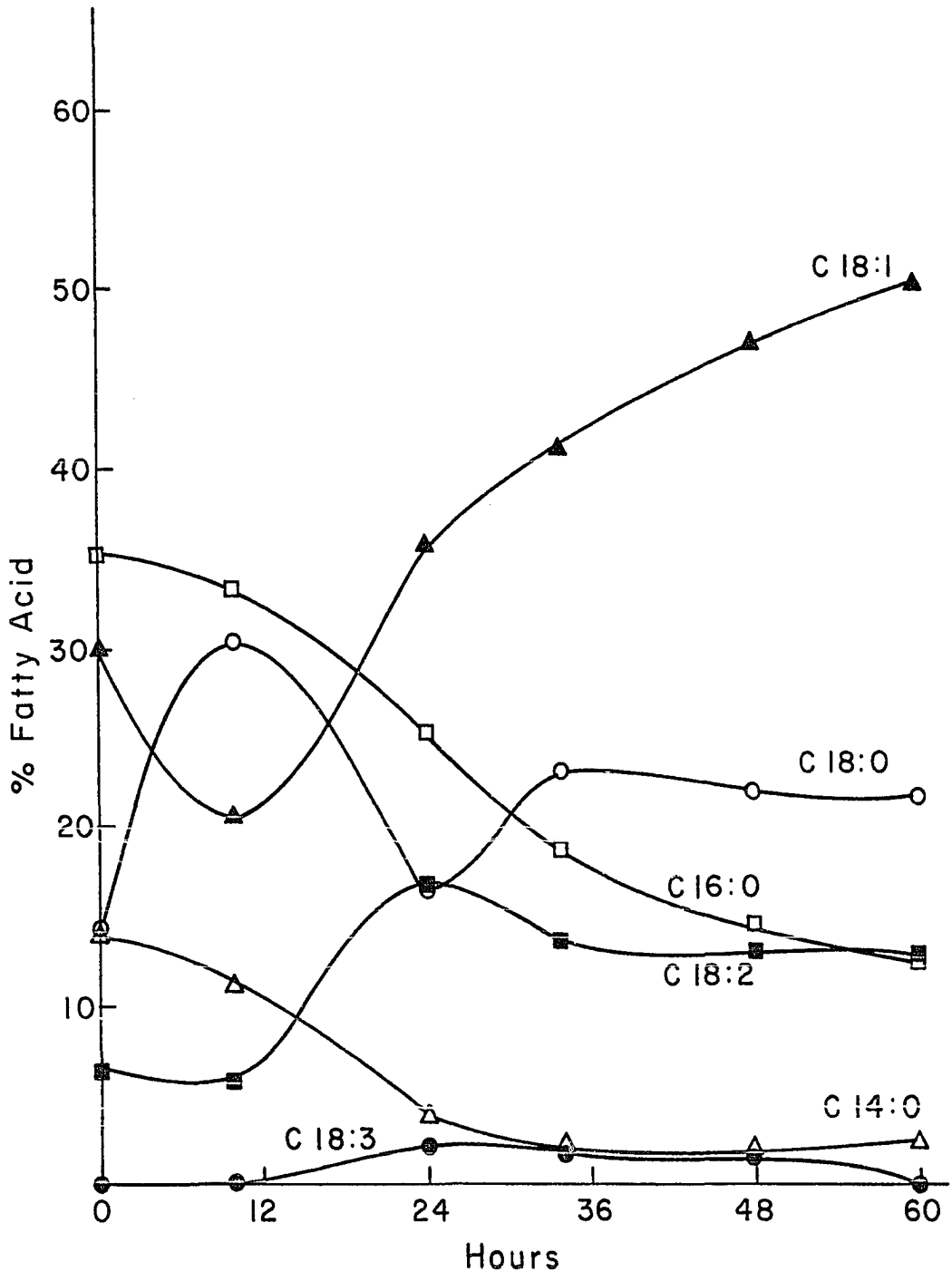


Figure 26. The change in fatty acid composition during the fermentation of whey by T. cutaneum 24. △ 14:0, □ 16:0, ○ 18:0, ▲ 18:1, ■ 18:2.

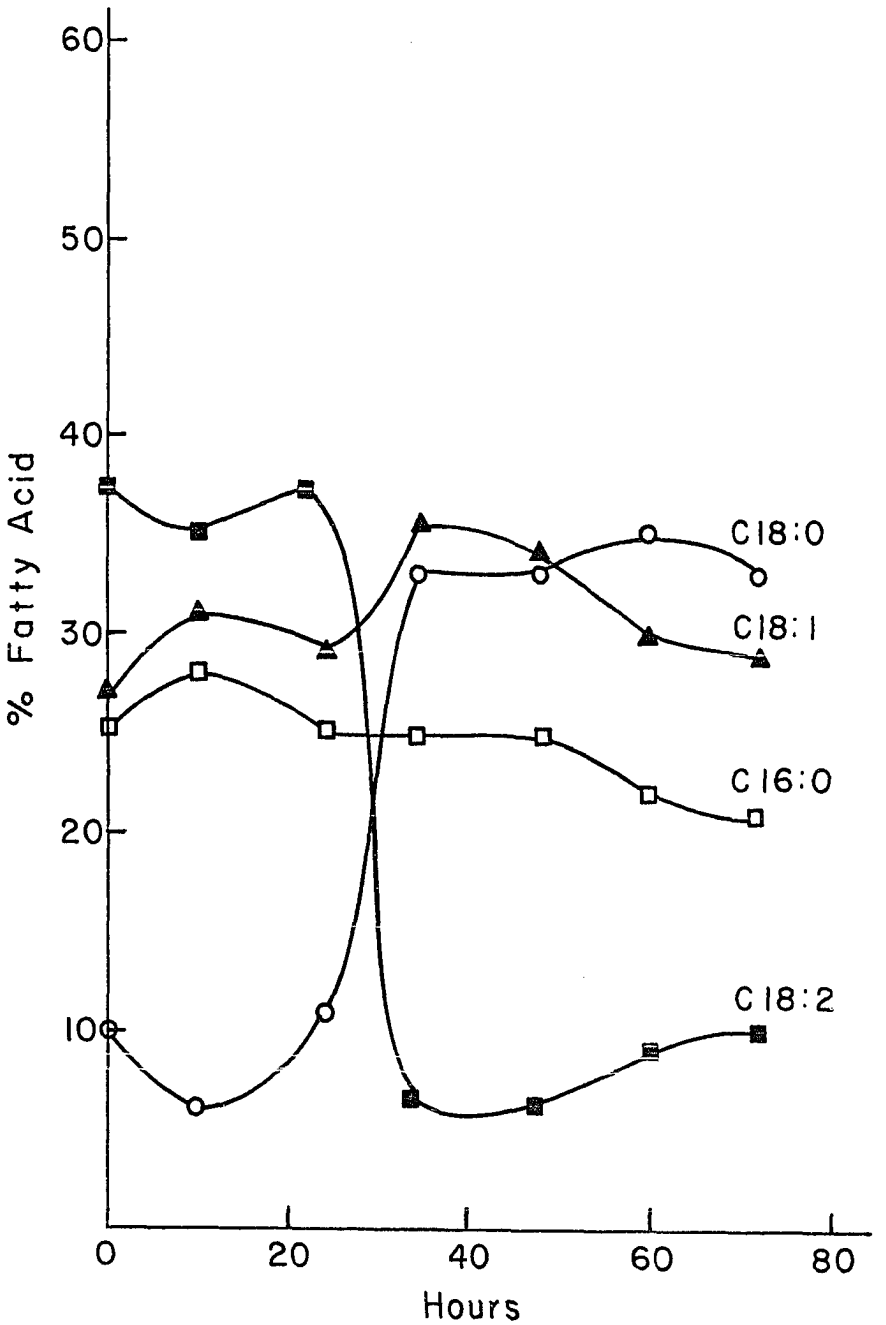


Figure 27. The change in fatty acid composition during the fermentation of whey permeate by T. cutaneum 24. □16:0, O18:0, ▲18:1, ■18:2.

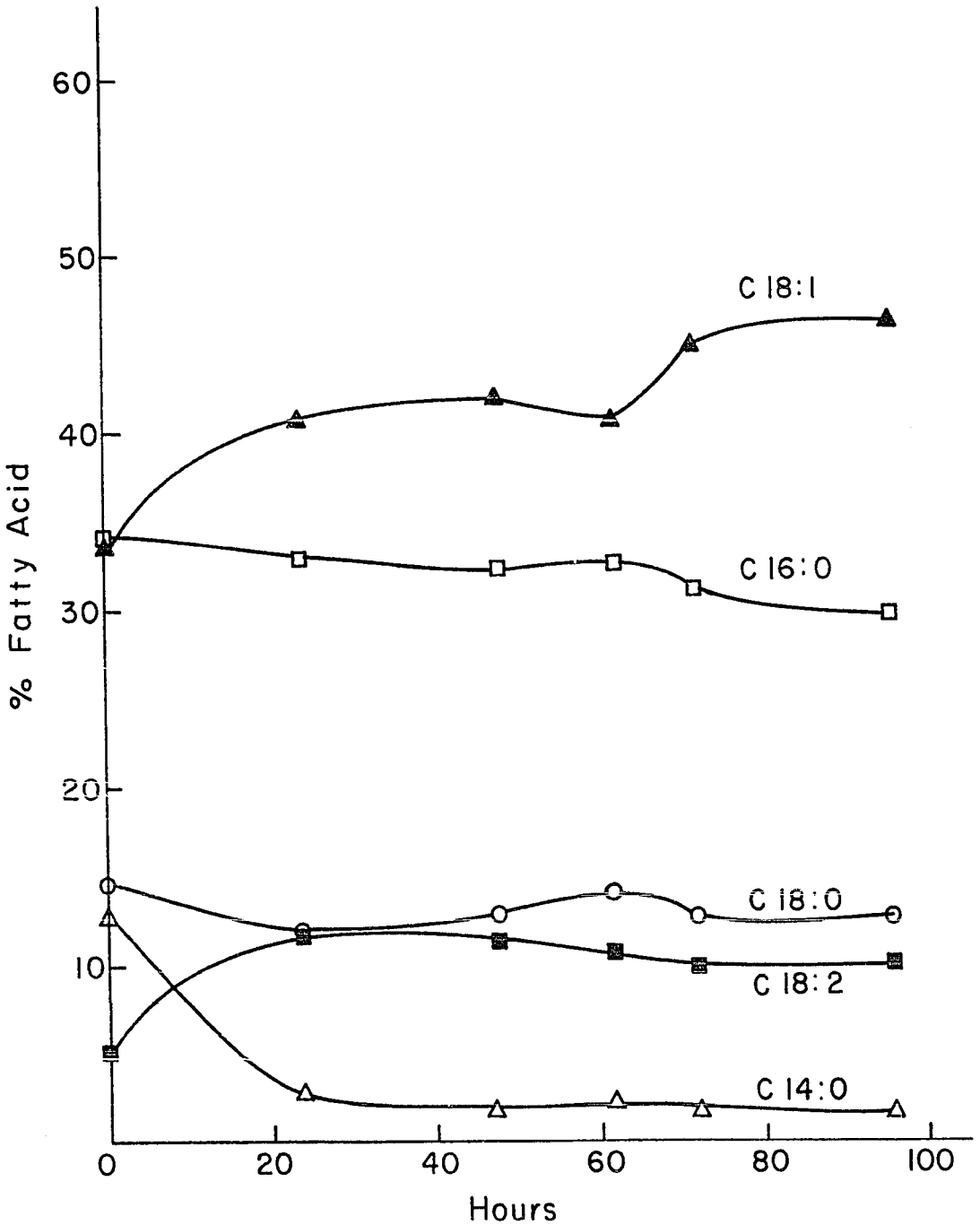


Figure 28. The change in fatty acid composition during the fermentation of whey by T. cutaneum 40. △14:0, □16:0, ○18:0, ▲18:1, ■18:2.

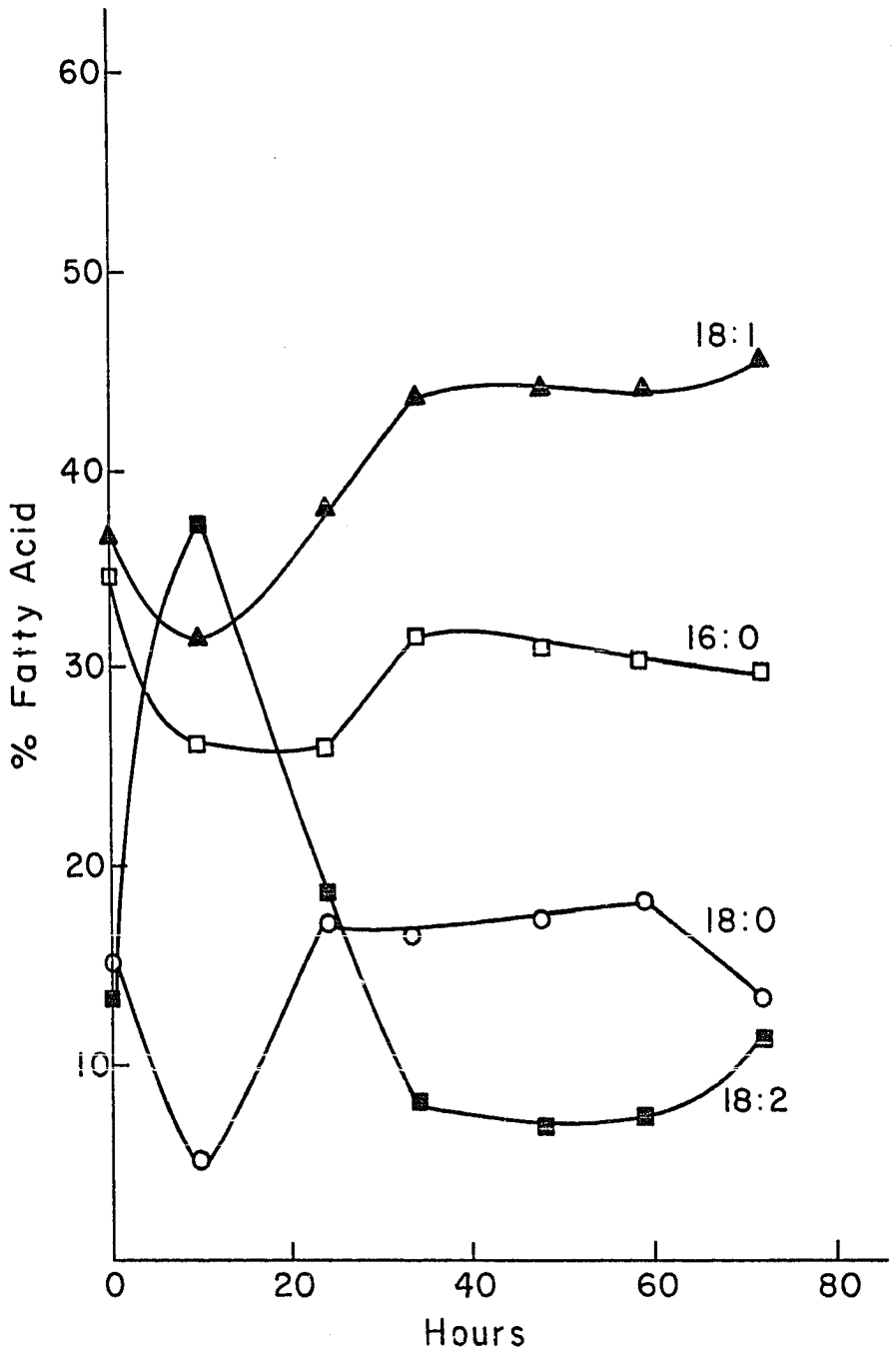


Figure 29. The change in fatty acid composition during the fermentation of whey permeate by T. cutaneum 40. □16:0, ○18:0, ▲18:1, ■18:2.

fat and render it liquid when its fatty acid composition would predict higher melting points.

The fat composition altered slightly when the organisms were grown in permeate as compared to whey. This may be due to the increased yield of fat alone as the triglyceride fatty acid composition is probably different than the membrane phospholipid composition. Phospholipids are generally more unsaturated than storage fat. The difference was most marked for T. cutaneum 24 which did not accumulate a high amount of fat. The fatty acid composition of this organism grown in permeate more closely resembles the composition at earlier incubation times in whey and at a similar fat yield.

C. curvata R did not markedly alter the fatty acid composition of its fat while growing on permeate. In contrast, the composition on whey showed increasing concentrations of oleic during the fermentation. It is possible that C. curvata R was well into the fattening phase when inoculated into the permeate and only continued to make fat. The % fat of the dry weight of this organism never really dropped to less than about 18% suggesting continual lipid accumulation (Table 10).

The fatty acid composition of the fat produced varied with the age of the culture. In the growth phase the degree of unsaturation and/or the content of shorter chained fatty acids was highest. This would indicate a low melting point and is probably indicative of the phospholipid composition. When fat production began, after about 10 hr, the relative proportion of $C_{18:1}$ dramatically increased while less change was observed in the other fatty acids. This may indicate increased deposition of triolein as

the cultures age although fatty acid analysis of triglycerides wasn't performed.

Amino Acid Composition

The amino acid composition of the cells produced is presented in Table 11. The nutritional value of a protein is often determined by its essential amino acid content with particular emphasis on lysine and methionine. The protein from the T. cutaneum strains is similar to other reported values (6) and compares favorably with the FAO/WHO (91) standard based on egg protein. The two C. curvata strains are low in lysine but compare favorably in other amino acids. The actual biological value of the single cell protein (SCP) can't be accurately determined from the amino acid composition alone. The digestibility of the protein and nucleic acid content should also be determined.

Table 11. Amino acid composition (g/100 g protein) of hydrolyzed cells of C. curvata strains R and D and T. cutaneum strains 24 and 40.

Amino acid	C. curvata		T. cutaneum		FAO/WHO[a]
	R	D	24	40	
Lysine	1.3	Tr[b]	5.5	5.5	5.5
Histidine	0.8	Tr	5.9	2.5	
Arginine	N.D.[c]	N.D.	N.D.	N.D.	
Aspartic acid	2.7	N.D.	Tr	N.D.	
Threonine	26.6	11.9	Tr	8.7	4.0
Serine	26.3	Tr	3.9	N.D.	
Glutamic acid	3.1	Tr	7.6	N.D.	
Proline	6.3	N.D.	Tr	N.D.	
Glycine	6.3	29.4	11.8	16.0	
Alanine	9.0	7.9	16.7	1.7	
Cysteine	N.D.	N.D.	N.D.	N.D.	
Valine	4.4	17.4	8.9	23.1	5.0
Methionine	2.5	9.3	7.7	5.7	3.0
Isoleucine	3.8	8.3	9.6	13.7	4.0
Leucine	7.0	15.7	22.4	23.0	7.0
Tyrosine	N.D.	N.D.	Tr	N.D.	
Phenylalanine	Tr	N.D.	Tr	N.D.	
Tryptophan	N.D.	N.D.	N.D.	N.D.	1.0
Total S	2.5	9.3	7.7	5.7	3.5
Total aromatic	Tr	Tr	Tr	Tr	6.0

[a]FAO/WHO recommended protein composition as reported by Young and Scrimshaw (91).

[b]Tr = Trace amounts detected (about 0.5 g/100 g protein).

[c]N.D. = Not detected (below about 0.5 g/100 g protein).

CONCLUSION

The fermentation of whey by these four organisms isolated from dairy plants is relatively inefficient because they are unable to use the whey proteins very well. The removal of these proteins prior to fermentation results in a much more efficient process. Under conditions for optimum growth and fattening, the most successful organism, C. curvata D, produced 149 g of fat in 72 hr from 568 g lactose. This process is reasonably rapid, requires only nitrogen addition and results in a spent medium low in soluble organic compounds. The cell mass also contains about 10% protein which appears to have a valuable amino acid composition.

The process might be improved economically by using nonsterile fermentations. Preliminary studies indicated that these organisms competed well in nonsterile whey. Large inocula might use the available nitrogen before other flora could grow very much.

The physical conditions of fermentation might also be altered so that the growth of the yeasts is favored. For example, increasing lactose concentrations to 20% in whey or permeate only slightly retarded the growth rates of these organisms and fattening occurred later in the fermentation. Other alterations such as suboptimal pH and temperature conditions might be used with some sacrifice in fermentation times.

REFERENCES

1. Ahearn, D. G., and W. E. Turner. 1970. Ecology and physiology of yeasts of an asphalt refinery and its watershed. Pages 113-123 in D. G. Ahern, ed. Recent trends in yeast research. Georgia State University Press, Atlanta, Ga.

2. Akin, C. 1973. Process for texturizing microbial cells by alkali-acid treatment. U.S. Patent 3,781,264.

3. Allen, L. A., N. H. Bernard, M. Fleming, and B. Hollis. 1964. Factors effecting the growth and fat formation of Rhodotorula gracilis. J. Appl. Bacteriol. 27:27-40.

4. American Public Health Association, Inc. 1967. Standard methods for the examination of water and waste water. 12th ed. American Public Health Association, Inc., New York, N.Y.

5. Andreyeva, L. N., and V. V. Biryukov. 1973. Analysis of mathematical models of the effect of pH on fermentation processes and their use for calculating optimal fermentation conditions. Pages 61-76 in B. Sikya, A. Prokop and M. Novák, eds. Advances in microbial engineering, Part 1. John Wiley and Sons, New York, N.Y.

6. Atkin, C., L. D. Witter, and Z. J. Ordal. 1967. Continuous propagation of Trichosporon cutaneum in cheese whey. Appl. Microbiol. 15: 1345-1351.

7. Barrow, A., E. G. Jefferys, R. H. J. Kessell, E. C. Lloyd, P. B. Lloyd, and I. S. Mixon. 1961. The metabolism of Gibberella fujikuroi in stirred culture. Can. J. Microbiol. 7:227-232.

8. Bernhauer, K. 1948. Studies on microbiological protein and fat synthesis. I. The basic requirements for protein and fat production in submerged culture. J. Rauch. Biochem. Z. B19:77-93. (Abstr.)

9. Bird, E. W. 1964. Laboratory exercises in dairy chemistry. Unpublished laboratory manual. Department of Food Technology, Iowa State University, Ames, Ia.

10. Bressani, R. 1968. The use of yeast in human foods. Pages 90-121 in R. I. Mateles and S. R. Tannenbaum, eds. Single cell protein. M.I.T. Press, Cambridge, Mass.

11. Brown, D. E. 1970. Aeration in the submerged culture of microorganisms. Pages 127-174 in J. R. Norris and D. W. Ribbons, eds. Methods in microbiology. Vol. 2. Academic Press, New York, N.Y.

12. Calloway, D. H. 1974. The place of SCP in man's diet. Pages 129-146 in P. David, ed. Single cell protein. Academic Press, New York, N.Y.

13. Castelli, A., G. P. Lettarrii, and G. Barbaresi. 1969. Effect of pH and CO_2 concentration changes on lipids and fatty acids of Saccharomyces cerevisiae. Arch. Mikrobiol. 66:34-39.

14. Chapman, D. 1975. Fluidity and phase transitions of cell membranes. Pages 1-7 in H. Eisenberg, E. Katchalski-Katzir, and L. A. Manson, eds. Biomembranes. Vol. 7. Plenum Press, New York, N.Y.

15. Chesters, C. G. C., and J. F. Peberdy. 1965. Nutritional factors in relation to growth and fat synthesis in Mortierella vinacea. J. Gen. Microbiol. 41:127-134.

16. Chio, J. S., and W. H. Peterson. 1953. Yeasts - methionine and cystine contents. Agric. Food Chem. 1:1005-1008.

17. Cieri, L., and F. Giodani. 1963. Beet molasses and the production of fat by microorganisms. Farmaci (Pavia) Ed. Pract. 18:592-595. (Abstr.)

18. Claydon, T. J., and R. M. Bechtle. 1971. Accelerated fermentation of cheese whey, developing the system. J. Dairy Sci. 54:1595-1604.

19. Collins, M. A., and B. W. Hammer. 1969. Fat hydrolysis. Pages 225-226 in V. B. D. Skerman, ed. Abstracts of microbiological methods. Wiley - Interscience Publishers, New York, N.Y.

20. Conway, E. J., and M. E. Beary. 1954. A magnesium-yeast and its properties. Biochem. J. 84:328-333.

21. Cronan, J. E., and E. P. Gelman. 1975. Physical properties of membrane lipids: Biological relevance and regulation. Bacteriol. Rev. 39:232-256.

22. Cullimore, D. R., and M. Woodbine. 1961. A superior fat-synthesizing single-spore strain of Lipomyces. Nature 190:1022-1024.

23. DeDeken, R. H. 1966. The crabtree effect: A regulatory system in yeasts. J. Gen. Microbiol. 44:149-156.

24. Dyr, J., and J. Protiva. 1964. Biosynthetic fat production by Rhodotorula gracilis in sulfite wastes. J. Sci. Food Agric. 15:1-214. (Abstr.)

25. Eisenberg, H., E. Katchalski-Katzir, and L. A. Manson, eds. 1975. Biomembranes. Plenum Press, New York, N.Y. 257 pp.

26. Enebo, L., L. G. Anderson, and H. Lundin. 1946. Microbiological fat synthesis by means of Rhodotorula yeast. Arch. Biochem. 11:383-395.

27. Fisher, L. J., and E. E. Lister. 1974. The inclusion of whey in rations for ruminants. Pages 23-30 in Proceedings of whey utilization symposium. Canadian Department of Agriculture, Ottawa, Ontario, Canada.

28. Folch, J., M. Lees, and G. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

29. Gad, A. M., and T. W. Walker. 1954. Mycological formation of fat. I. Media conducive of fat formation from sucrose by Aspergillus nidulans, Penicillium javanicum and Penicillium spinulosum. J. Sci. Food Agric. 7:339-343.

30. Ginsburg, A., and E. R. Stadtman. 1970. Multienzyme systems. Annu. Rev. Biochem. 39:429-472.

31. Gregory, M. E., and M. Woodbine. 1953. Microbial synthesis of fat. J. Exp. Bot. 4:314-318.

32. Greshnykh, K. P., A. N. Grigoryan, E. M. Dekanskaya, É. V. Dyatlovitskaya, and L. D. Bergel'son. 1968. Effects of temperature and nitrogen source on lipid biosynthesis by yeast grown on n-alkanes. Microbiology 37:208-210.

33. Hayashi, E., R. Hasegawa, and T. Tomita. 1976. Accumulation of neutral lipids in Saccharomyces carlsbergensis by myo-inositol deficiency and its mechanism. J. Biol. Chem. 251:5759-5769.

34. Herbert, D., R. Elsworth, and R. C. Telling. 1956. The continuous culture of bacteria; a theoretical and experimental study. J. Gen. Microbiol. 14:601-622.

35. Hettinga, D. H., A. H. Miah, E. G. Hammond, and G. W. Reinbold. 1970. Sensitive enzymatic method for determination of glucose, galactose, and lactose in cheddar cheese. J. Dairy Sci. 53:1377-1380.

36. Höfer, M., K. Brand, K. Deckner, and J. U. Becker. 1971. Importance of the pentose phosphate pathway for D-glucose catabolism in the obligatory aerobic yeast Rhodotorula gracilis. Biochem. J. 123: 855-863.

37. Hunter, K., and A. H. Rose. 1971. Yeast lipids and membranes. Pages 211-270 in A. H. Rose and J. S. Harrisen, eds. The yeasts. Vol. II. Academic Press, London.

38. Hunter, K., and A. H. Rose. 1972. Lipid composition of Saccharomyces cerevisiae as influenced by growth temperature. Biochim. Biophys. Acta 260:639-653.

39. Johnson, B., and C. M. Brown. 1972. A possible relationship between the fatty acid composition of yeasts and the 'petite' mutation. Antonie van Leeuwenhoek J. Microbiol. Serol. 38:137-144.

40. Johnson, M. G., J. Borkowski, and C. Engblom. 1964. Steam sterilizable probes for dissolved oxygen measurement. Biotechnol. Bioeng. 6:457-468.

41. Kaneko, H., M. Hosohara, M. Tanaka, and T. Stoh. 1976. Lipid composition of 30 species of yeast. Lipids 11:837-844.

42. Kates, M., and R. M. Baxter. 1962. Lipid composition of mesophilic and psychrophilic yeasts (Candida species) as influenced by environmental temperatures. Can. J. Biochem. Physiol. 40:1213-1227.

43. Khan, A. W., and T. K. Walker. 1961. Fat production from cane molasses by Penicillium spinulosum: A study on substances in cane molasses interfering with fat production. Can. J. Microbiol. 7: 895-899.

44. Kihlberg, R. 1972. The microbe as a source of food. Annu. Rev. Microbiol. 26:427-466.

45. Krumphanzl, V., V. Grégr, J. Pelechová, and J. Uher. 1973. Biomass and fat production in Rhodotorula gracilis. Biotechnol. Bioeng. Symp. No. 4:245-256.

46. Ladbrooke, B. D., and D. Chapman. 1969. Thermal analysis of lipids, proteins and biological membranes: A review and summary of some recent studies. Chem. Phys. Lipids 3:304-367.

47. Lemoigne, M., J. P. Aubert, and J. Miller. 1954. La production d'alcoal et le rendement de croissance de la levure de boulangerie cultivée en aérobiöse. Annu. Inst. Pasteur 87:427.

48. Lewin, L. M. 1965. Effects of meso-inositol deficiency on some important biological and chemical characteristics of yeast. J. Gen. Microbiol. 41:215-224.

49. Lipinsky, E. S., and J. H. Litchfield. 1970. Algae, bacteria and yeasts as food or feed. C.R.C. Crit. Rev. in Food Technol. 1:581-618.

50. Lodder, J. 1970. The yeasts. North Holland Publishing Co., Amsterdam, the Netherlands. 1385 pp.

51. Lodge, G. A., and J. I. Elliot. 1974. Monogastric feeding of whey. Pages 31-40 in Proceedings of whey utilization symposium. Canadian Department of Agriculture, Ottawa, Ontario, Canada.

52. Luddy, F. E., R. A. Barford, S. E. Hert, and P. Magidman. 1968. A rapid and quantitative procedure for the preparation of methyl esters of butter, oil and other fats. J. Am. Oil Chem. Soc. 45:549-552.

53. Lynen, F. 1968. The fatty acid synthetase from yeast. Pages 271-299 in A. K. Wills and H. Krebs, eds. Aspects of yeast metabolism. F. A. Davis Co., Philadelphia, Pa.

54. McElwain, M. D., T. Richardson, and C. H. Amundsen. 1975. Some functional properties of succinylated single cell protein concentrate. J. Milk Food Technol. 38:521-526.

55. Mateles, R. I., and E. Battat. 1974. Continuous culture used for media optimization. Appl. Microbiol. 28:901-905.

56. Meyer, S. A., and H. J. Phaff. 1970. Taxonomic significance of the DNA base composition in yeasts. Pages 1-29 in D. G. Ahearn, ed. Recent trends in yeast research. Georgia State University Press, Atlanta, Ga.

57. Mickle, J. B., W. Smith, D. Halter, and S. Knight. 1974. Performance and morphology of Kluyveromyces fragilis and Rhodotorula gracilis grown in cottage cheese whey. J. Milk Food Technol. 37:481-484.

58. Mumma, R. O., C. L. Feigus, and R. D. Sekura. 1970. The lipids of thermophilic fungi: Lipid composition comparisons between thermophilic and mesophilic fungi. Lipids 5:100-103.

59. Naguib, K., I. A. Al-Sohaily, and A. S. Al-Saultan. 1972. On mycological fat production from Araqi date extract. Mycopathol. Mycol. Appl. 47:93-103.

60. Naguib, K., and E. S. Yassa. 1974. The effect of magnesium and phosphate on mycological fat formation from sweet potatoes. Mycopathol. Mycol. Appl. 52:177-185.

61. Osman, H. G., M. Abdel-Akher, A. M. H. El-Refai, and M. A. Nashat. 1969. The biosynthesis of fat by Penicillium lilacinum. Z. Allg. Mikrobiol. 9:283-285.

62. Oura, E. 1974. Effect of aeration intensity on the biochemical composition of baker's yeast. I. Factors affecting the type of metabolism. Biotechnol. Bioeng. 16:1197-1212.

63. Pan, S. C., A. A. Andreasen, and P. Kolachov. 1949. Factors influencing fat synthesis by Rhodotorula gracilis. Arch. Biochem. 23: 419-433.

64. Perlman, D., and C. J. Sih. 1960. Fungal synthesis of citric, fumaric and itaconic acids. Pages 167-194 in D. J. D. Hokenhull, ed. Progress in industrial microbiology. Vol. 2. Interscience Publishers, Inc., New York, N.Y.

65. Phillips, K. L. 1963. Determination of oxygen and carbon dioxide using a paramagnetic oxygen analyzer. Biotechnol. Bioeng. 5:9-15.

66. Prescott, S. C., and C. G. Dunn. 1940. Fat production by yeasts and yeast-like microorganisms. Pages 425-433 in Industrial microbiology. 1st ed. McGraw-Hill Book Co., New York, N.Y.

67. Prill, E. A., P. R. Wenck, and W. H. Peterson. 1935. The chemistry of mold tissue. VI. Factors influencing the amount and nature of the fat produced by Aspergillus fischeri. Biochem. J. 29:22-33.

68. Rasmussen, R. K., and H. P. Klein. 1968. Activation of fatty acid synthesis in cell-free extracts of Saccharomyces cereviseae. J. Bacteriol. 95:157-161.

69. Rattray, J. B. M., A. Schibeci, and D. Kidley. 1975. Lipids of yeasts. Bacteriol. Rev. 39:197-231.

70. Rohde, P. A., ed. 1967. BBL manual of products and laboratory procedures. Bioquest, Cockeysville, Md. 210 pp.

71. Seeley, R. D. 1975. Functional aspects of SCP - are a key to potential markets. Food Process. Dev. 9:46-51.

72. Shannon, L. J., and K. E. Stevenson. 1975. Growth of fungi and BOD reduction in selected brewery wastes. J. Food Sci. 40:826-829.

73. Snyder, H. E. 1970. Microbial sources of protein. Adv. Food Res. 18:85-140.

74. Sobus, M. T., and C. E. Holmlund. 1976. Extraction of lipids from yeasts. Lipids 11:341-348.

75. Starkey, R. L. 1946. Lipid production by a soil yeast. J. Bacteriol. 51:33-50.

76. Steinberg, M. D., and Z. J. Ordal. 1954. Microbiological fat production, effect of fermentation variables on rate of fat formation by Rhodotorula gracilis. Agric. Food Chem. 2:873-877.

77. Sterkin, V. E., I. M. Chirkov, and V. A. Samoylenko. 1973. Study of transitional stages in continuous culture of microorganisms. Pages 53-60 in B. Sikya, A. Prokop, and M. Novák, eds. Advances in microbial engineering. Part 1. John Wiley and Sons, New York, N.Y.

78. Stewart, R. R. 1975. Analytical methods for yeasts. In D. M. Prescott, ed. Methods in cell biology. Vol. 2. Yeast cells. Academic Press, New York, N.Y.

79. Stout, J. D. 1972. Response of some soil yeasts and bacteria to glucose. Soil Biol. Biochem. 4:533-536.

80. Tien, H. T. 1974. Bilayer lipid membranes (BLM). Marcel Dekker, Inc., New York, N.Y. 655 pp.

81. U.S. Department of Agriculture Statistical Reporting Service. Crop reporting board. 1975. Dairy products annual summary No. Da 2-1(76). Washington, D.C.

82. Vagelos, P. R. 1971. The regulation of fatty acid biosynthesis. Pages 119-166 in B. L. Horecker and E. R. Stadtman, ed. Current topics in cellular regulation. Vol. 4. Academic Press, New York, N.Y.

83. Vananuvat, P., and J. E. Kinsella. 1975. Some functional properties of protein isolates from yeast, Saccharomyces fragilis. J. Agric. Food Chem. 23:613-616.

84. van Deenan, L. L. M. 1965. Phospholipids and biomembranes. Pages 1-115 in R. T. Holman, ed. Progress in the chemistry of fats and other lipids. Vol. 8, part 1. Pergamon Press, New York, N.Y.

85. Webb, B. H., and E. O. Whittier. 1970. By-products from milk. AVI Publishing Co. Inc., Westport, Conn. 428 pp.

86. Webb, B. H., A. H. Johnson, and J. A. Alford. 1974. Fundamentals of dairy chemistry. 2nd ed. AVI Publishing Co., Westport, Conn. 929 pp.

87. Weete, J. D. 1974. Fungal lipid biochemistry. Plenum Press, New York, N.Y. 393 pp.

88. Weiss, T. J. 1970. Food oils and their uses. The AVI Publishing Co., Westport, Conn. 224 pp.

89. Whitworth, D. A., and C. Ratledge. 1975. An analysis of intermediary metabolism and its control in a fat synthesizing yeast (Candida 107) growing on glucose or alkanes. J. Gen. Microbiol. 88: 275-288.

90. Woodbine, M. 1959. Microbial fat: Micro-organisms as potential fat producers. Prog. Ind. Microbiol. 1:181-245.

91. Young, V. R., and N. S. Scrimshaw. 1975. Clinical studies on the nutritional value of single cell proteins. Pages 564-586 in S. R. Tannenbaum and D. I. C. Wang, eds. Single cell protein. II. M.I.T. Press, Cambridge, Mass.

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APPENDIX

Table 12. Characteristics of selected yeasts and molds from Northern Regional Research Laboratories, Peoria, Ill.

	Proteolytic	Lipolytic	Lactose utilization	Fat production
Yeasts				
Lipomyces lipofer Y-1394	+	-	+	+
Lipomyces starkeyi Y-1389	-	-	+	+
Rhodotorula glutins Y-1091	+	-	+	+
Endomyces vernalis YB-4325	-	-	±	+
Molds				
Geotrichum candidum 7366	-	+	+	+
Fusarium oxysporium 1943	+	-	+	-
Fusarium lini 2204				-
Penicillium spinulosum 724	-	+	+	-
Aspergillus medulans 187	-	+	+	-
Penicillium javanicum 707	-	-	±	
Aspergillus flavis 1957	-	+	+	-
Aspergillus flavipes 295	-	-	+	-
Penicillium pescarium 1075	-	+	±	
Fusarium bulbigenum var. *lycopersici* 1985	-	+	+	-
Mucor plumbeus 1432	-	+	+	-
Mucor mucedo 3634	+	+	+	-
Candida utilis Y-900				
Mucor ramanneanus 1296	-	+	+	-
Mucor circinelloides 3614	+	+	+	-
Mucor albo-ater 3318	+	+	+	-

Table 13. Characteristics of selected yeasts and molds from the culture collection[a] of Iowa State University Botany Department.

	Proteolytic	Lipolytic	Lactose utilization	Fat production
Mucor T-3	+	+	+	-
Mucor T-2	-	+	+	-
Mucor T-1	-	+	+	-
Mucor T-5	-	+	+	-
Geotrichum sp.	-	+	+	-
Helmenthosporium sp.	-	+	+	-
Aspergillus niger	-	+	+	-
Mycogne sp.	+	-	+	±
Mucor F16	-	+	+	-
Trichorus sp.	+	+	+	-
Aspergillus flavis	-	+	+	-
Scopulariorisis sp.	-	-	+	-
Curvalaria sp.	-	-	+	-
Fusarium sp. 169	+	-	+	-
Penicillium sp. 62	+	+	±	-
Botrytes sp.	+	+	±	-
Cladasporium sp.				-
Trichoderma sp.	+	+	+	-
Aspergillus clavatus	-	+	+	-
Penicillium sp. 11E	+	-	+	-
Fusarium sp. 19, 5.6				-

[a]Cultures supplied by Dr. Lois Tiffany.

Table 14. Characteristics of yeasts isolated from environmental samples and two cheese plants.

Number	Source	Proteolytic	Lipolytic	Lactose utilization	Fat production
A	Milk	+	+	+	-
B	Floor drain	+	-	+	+
C	Floor drain	-	-	+	-
D	Floor drain	+	-	+	+
E	Tree stump	+	-	-	-
F	Forest soil	-	+	+	-
G	Soil Lake LaVerne	-	+	+	-
H	Standing water	-	+	+	-
I	Soil	-	+	-	-
J	Tree stump	-	-	+	-
K	Horse manure	-	+	+	-
L	Forest edge soil	+	+	-	-
M	Soil Lake LaVerne	-	+	-	-
N	Floor drain	-	-	+	-
O	Soil Lake LaVerne	-	-	-	-
P	Horse manure	-	+	+	-
Q	Forest edge	-	+	+	-
R	Floor drain	±	+	+	+
1[a]	Sewage drain S.C.[b]	-		±	+
2	Sewage drain S.C.	-		±	-
3	Whey drain S.C.	-		-	-
4[a]	Whey drain S.C.	-		+	+
5	Whey drain S.C.	-		±	-
6	Whey drain S.C.	-		+	-
7[a]	Sewage drain S.C.	-		+	+

[a]Most promising organisms in terms of fattening and growth. Placed in group of organisms with similar morphology. Group 1 = isolates 1, 40, 7, 32. Group 2 = isolates 24, 19, 17. Group 3 = isolates 25, 4. Group 4 = isolates 37, 10.

[b]State Center Farmers Cooperative Association, State Center, Ia.

Table 14. Continued.

Number	Source	Proteolytic	Lipolytic	Lactose utilization	Fat production
8	Sewage drain S.C.	-		±	+
9	Sewage drain S.C.	-		+	+
10[a]	Sewage drain S.C.	-		+	-
11	Whey drain mold S.C.	-		+	-
12	Whey drain mold S.C.	+		-	-
13	Whey drain mold S.C.	-		+	-
14	Whey drain mold S.C.	-		+	-
15	Whey tank Newton[c]	-		+	-
16	Whey tank Newton	-		±	-
17[a]	Floor swab Newton	-		+	+
18	Separator drain Newton	-		+	+
19[a]	Whey drain Newton	-		+	+
20	Whey drain Newton	-		-	-
21	Whey drain Newton	-		-	-
22	Whey drain Newton	-		-	-
23	Whey drain Newton	-		-	-
24[a]	Floor swab Newton	-		+	+
25[a]	Floor swab Newton	-		+	+
26	Floor swab Newton	-		+	+
27	Floor swab Newton	-		-	-
28	Whey tank Newton	-		-	-
29	Whey tank Newton	-		-	-
30	Whey tank Newton	-		-	-
31	Whey tank Newton	-		-	-
32	Truck wash	-		-	-
33	Truck wash	-		-	-
34	Whey tank	-		-	-
35	Whey tank	-		-	-
36	Whey drain S.C.	+		-	-

[c]Maytag Dairy Farms Inc., Newton, Ia.

Table 14. Continued.

Number	Source	Proteolytic	Lipolytic	Lactose utilization	Fat production
37[a]	Sewage drain S.C.	-		+	+
38	Sewage drain S.C.	-		-	-
39[a]	Sewage drain S.C.	-		-	+
40[a]	Sewage drain S.C.	-		+	+
41	Whey tank Newton	-		-	-
42	Whey tank Newton	-		-	-