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RELATIVE REACTIVITIES OF SUGARS IN THE FORMATION OF 5-HYDROXYMETHYLFURFURAL IN SUGAR-CATALYST MODEL SYSTEMS[1]

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ABSTRACT

Model systems were employed to determine the relative reactivity of sucrose, glucose, and fructose in the formation of 5-hydroxymethylfurfural (5-HMF) at pH 3.5. Fructose was the major reactant for formation of 5-HMF. Fructose was 31.2 times faster than glucose, whereas sucrose was 18.5 times faster than glucose in the rate of 5-HMF formation when averaged over three different sugar-catalyst systems. Accelerating effects of citric acid, minerals (calcium, magnesium, and potassium), and amino acids (alanine, aspartic acid, and γ-aminobutyric acid) in the formation of 5-HMF from sugars were evaluated. With fructose as the substrate, 5-HMF formed 5 times faster in the presence of citric acid and minerals than in the presence of HCl. Varying catalytic effects were noted with the three amino acids. Rates of 5-HMF formation from glucose and sucrose showed slight enhancement in the presence of the amino acids, whereas virtually no enhancement occurred when fructose was the substrate.

INTRODUCTION

In a previous storage study of grapefruit juice (Lee and Nagy 1988a), sucrose was virtually degraded during storage for 9 weeks at 40°C, and the relative percentage loss of fructose and glucose suggested the contribution of reducing sugars to the browning of stored grapefruit juice. Another major trend observed was that the concentration of 5-hydroxymethylfurfural (5-HMF) significantly increased during storage. It is well established that decomposition of hexoses yield 5-HMF. However, it is difficult to ascertain a stoichiometric relationship

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between sugar loss and 5-HMF formation because the observed accumulation of 5-HMF might result from equilibrium conditions between formation and degradation; the mechanisms of which are not clearly known. The rate of formation of 5-HMF was observed as a function of temperature and soluble solids content (Toribio and Lozano 1987). Wucherpfennig and Burkardt (1983) also determined that the concentration and type of sugars, quantity and structure of amino acids, as well as pH, additively effect 5-HMF formation. 5-HMF is known as a precursor of browning and its close relationship to the color deterioration in stored grapefruit juice has been well documented (Lee and Nagy 1988b). In the present work, we compare the relative reactivities of sugars in the formation of 5-HMF using model systems simulating grapefruit juice. An understanding of the mechanisms of 5-HMF formation should enhance our knowledge of the role of this important reactive chemical in nonenzymic browning.

MATERIALS AND METHODS

Materials

Sugars (glucose, fructose, and sucrose), amino acids (alanine, aspartic acid, γ-aminobutyric acid), citric acid, HCl, and NaOH were commercially available (Fisher Scientific Co.).

Model Systems

Model systems were prepared similar to the chemical composition of grapefruit juice as reported in the literature (Nagy *et al.* 1977). Model systems consisted of (1) 0.5 M solution of sugars and 0.05 M citric acid at pH 3.5, (2) 0.5 M sugars and 0.05 M HCl at pH 3.5, (3) 0.5 M sugars plus 0.05 M citric acid plus 0.15% KCl, 0.0065% $CaCl_2$ and 0.01% $MgCl_2$ at pH 3.5, (4) 0.5 M sugars plus 0.05 M citric acid and 0.05 M amino acids at pH 3.5. The pH of all solutions was adjusted with 2 N NaOH or 0.2 N HCl. Twenty-five portions of the solutions were distributed into screw-cap test tubes (Kimex, Fisher Scientific Co.) in duplicate, sealed fingertight, and held in a laboratory oven at 50°C. Tubes were periodically removed for analyses during the experiment.

5-HMF Measurements

5-HMF was determined by HPLC. HPLC analysis was carried out with a Waters Model 600E gradient pump, LDC/Milton Roy variable wavelength detector (UV 280 nm), Shimadzu Model SIL-6A autoinjector/system controller and Shimadzu C-R3A data processor. A Zorbax ODS column (4.6 mm, i.d. × 250 mm), and an RP-18 guard column (4.6 mm, i.d. × 30 mm) were used. A linear gradient and isocratic elution with 1% acetic acid/water (solvent A) and

1% acetic acid/acetonitrile (solvent B) was used at a flow rate of 1 ml/min: initial, 95% A, 5% B; 5–25 min, linear gradient, 95 to 90% A, 5 to 10% B. Finally, the column was washed with 100% B for 5 min, and equilibrated with 95% A and 5% B for 10 min. The sample was filtered through 0.45 μm HV type filter from Waters (Milford, MA) and 10 μL was injected. Each sample was injected three times.

Statistical Analysis

The data were analyzed using regression analyses procedure available on the Minitab statistical package accessible on the VAX 11/750 computer at the CREC.

RESULTS AND DISCUSSIONS

Relative Reactivities of the Sugars

Formation of 5-HMF was quantified by HPLC to compare the relative reactivity of sugars under similar experimental conditions. The reproducibility of the method was determined by analyzing six runs of sample; the coefficient of variation was 3.6%. Figure 1 shows linear regression models for the formation

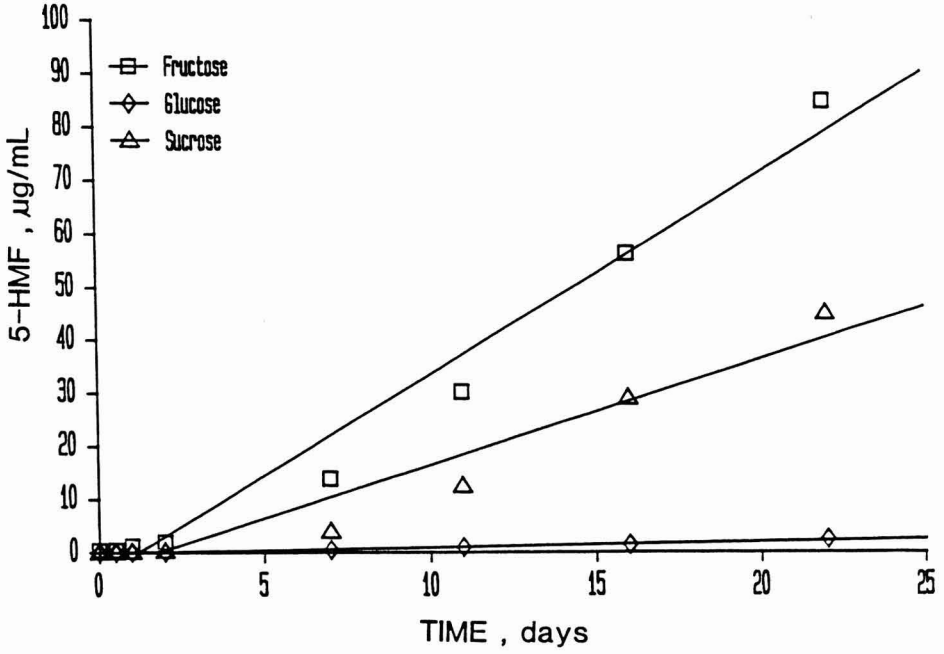

FIG. 1. LINEAR REGRESSION ANALYSIS OF THE FORMATION OF 5-HMF IN SUGAR-CITRIC ACID MODEL SYSTEMS AS A FUNCTION OF TIME AT 50°C AND pH 3.5

of 5-HMF from sugars (Model 1) as a function of time. 5-HMF formed rapidly from fructose; almost 0.47 μg/mL formed within 1 day and after 22 days, this increased to over 84.4 μg/mL. However, glucose was not as rapid in the formation of 5-HMF. Less than 0.04 μg/mL was formed after 1 day and less than 2.5 μg/mL formed after 22 days. The amount of 5-HMF from fructose was over 33 times that from glucose after 22 days at 50°C. No 5-HMF was detected during the early stage of reaction with sucrose, but levels readily reached those found with glucose within 12 h. The amount of 5-HMF from sucrose was about 0.07 μg/mL after 1 day, and after 22 days this increased to 44.8 μg/mL. Sucrose, a nonreducing sugar, does not undergo the sugar dehydration reaction until it is hydrolyzed to the reducing sugars (inversion). When hydrolysis conditions lead to glucose and fructose, then these compounds subsequently undergo sugar degradation reactions. Sucrose has a potential reducing sugar concentration equivalent to the combined total of glucose and fructose. It generates more 5-HMF than the glucose system, but less than fructose. Formation of 5-HMF is recognized as a primary breakdown product during the dehydration of glucose or fructose in an acid medium (Singh *et al.* 1948).

To compare the relative rates of 5-HMF formation from the 3 sugars (Fig. 1), rate constants for formation of 5-HMF were calculated using linear regression equations. For fructose, regression analysis yielded the equation: 5-HMF content $= -4.519 + 3.779$ (days) [or $y = -4.519 + 3.779x$] with $R^2 = .973$. The glucose model yielded: $y = -0.089 + .104x$ ($R^2 = .977$) while sucrose yielded: $y = -3.502 + 1.989x$ ($R^2 = .940$). Relative rates were calculated by assuming the rate constant for glucose equal to 1. Fructose was about 31 times faster than glucose, and sucrose was about 18 times faster than glucose in the rate of 5-HMF formation over 22 days of storage under 3 different sugar-catalyst systems (Table 1). Fructose was the most reactive sugar among the three sugars in the formation of 5-HMF under the experimental conditions. In a previous comparison of the stabilities of glucose and fructose at different pHs (Shallenberger and Mattick 1983), fructose was not stable in the environment of pH 4–6. Fructose is 5 times more reactive than glucose in its most stable acidic environment (pH 2–6). Conformational stability of sugar molecules may explain the faster rate of 5-HMF formation from fructose than glucose because fructose enolizes faster than glucose (Isbell *et al.* 1969). Formation of the intermediate 1,2-enediol form is the primary step and, probably, the rate-determining step in the formation of 5-HMF from hexoses. Enolized sugar can easily be dehydrated and fragmented to produce 5-HMF. In the case of sucrose, however, the situation is somewhat more complicated owing to a pre-step of acid-catalyzed hydrolysis of the molecule. Since fructose produces 5-HMF much faster than glucose, the rate of 5-HMF formation from sucrose was most likely due to the fructose portion of the molecule.

TABLE 1.
RELATIVE RATES FOR FORMATION OF 5-HMF FROM SUGARS (0.5 M) AT pH 3.5[1]

Sugars	HCl	Citric	Citric + K^+, Ca^{2+}, Mg^{2+}	Mean
Glucose	1.0	1.0	1.0	1.0
Fructose	21.6	36.0	35.9	31.2
Sucrose	15.7	18.9	21.0	18.5

[1]($K_{sugar}/K_{glucose}$); K = rate constant = μg 5-HMF/day

Effects of Catalysts

To evaluate the effects of acid type and minerals on 5-HMF formation, two model systems (Models 2 and 3) were employed. Table 2 compares the rate constants and relative rates of HCl, citric acid, and citric acid plus minerals on the formation of 5-HMF from fructose. HCl was not an effective catalyst for the formation of 5-HMF from fructose; however, the minerals (Ca^{2+}, Mg^{2+}, K^+) showed catalytic effects. With citric acid, the reaction was about 4.9 times more rapid than with HCl, and with the additional presence of some minerals, it was about 5.0 times as rapid compared to HCl alone (Table 2). Similar trends were observed for glucose and sucrose. The rate of formation of 5-HMF from fructose with minerals was only 1.04 times more rapid than fructose without minerals (Table 2). Large amounts of organic acids can create favorable conditions for degrading sugars by promoting enolization of those reducing sugars. Thus, the accelerating effects of carboxylated anions, such as citric and malic acids, their salts, and some minerals in grapefruit juices on the degradation of sugars should be considered during processing and subsequent storage. Similar accelerating effects of phosphates and other buffers containing carboxylate anions on sugar decomposition have been observed (Kato *et al.* 1969).

The data from our model systems show that sugar, per se, decomposes to reactive compounds under moderate acidic conditions without any necessity for direct interaction by amino groups. However, free amino acids are present in citrus juices at about 200–400 mg/100 mL, and there is continuing debate as to the relative importance of amino acids in the browning of citrus products. To this end, we were interested in studying the effects of amino acids on the formation of 5-HMF from sugars. Three amino acids such as alanine, aspartic

TABLE 2.
RATES OF FORMATION OF 5-HMF FROM FRUCTOSE (0.5 M) AT pH 3.5

Catalyst	Rate Constant[1]	r^2	Relative Rate
HCl	0.776	0.9860	1.0
Citric	3.779	0.9866	4.9
Citric + K^+, Ca^{2+}, Mg^{2+}	3.913	0.9875	5.0

[1]K = μg 5-HMF/day
[2]Coefficient of correlation

acid, and γ-aminobutyric acid were chosen as they are some of the most abundant amino acids naturally occurring in citrus juices (Nagy *et al.* 1977).

Table 3 summarizes the relative rate of formation of 5-HMF from sugars with amino acids. There were slight differences in the rate of 5-HMF formation from sugars; the rate of 5-HMF formation from glucose was slightly enhanced by the presence of amino acids. The rate of 5-HMF formation from sucrose was also enhanced, possibly suggesting that sucrose hydrolysis was catalyzed by the presence of amino acids. It was interesting, however, to note that there were no measurable differences in the rate of 5-HMF formation from fructose with added amino acids. This effect is in contrast to glucose reactivity. Coincidentally, McWeeny (1973) observed that the loss of amino acids or free amino groups was very low with fructose, as compared to glucose. Buera *et al.* (1987) also observed a small change in the activation energy of browning in the reaction between fructose and glycine as compared to glucose and glycine. Those workers suggested that fructose contained a high percentage of the acyclic form, thus, the catalytic effects of the amino acid were less important. We are not certain why fructose appeared to have different rate-determining steps or different mechanisms to formation of 5-HMF when contrasted to glucose in the presence of amino acids.

Relative reactivities of sugars with amino acids may be applicable only to the particular conditions employed. In some model studies (Kato *et al.* 1969; Reyes *et al.* 1982), reactivity of fructose was greater than glucose when a lower concentration of amino acid was employed, and/or during early stages of the reaction sequence. The situation was reversed at higher concentrations of amino acid and/or at a later stage of the reaction. Catalytic effects of amino acids have been

TABLE 3.
RELATIVE RATES FOR THE FORMATION OF 5-HMF FROM SUGARS WITH AMINO ACIDS AND/OR CITRIC ACID AT pH 3.5[1]

	Glucose	Fructose	Sucrose
Citric	1.00	1.00	1.00
Citric + Alanine	1.08	0.96	1.09
Citric + Aspartic	1.15	0.98	1.10
Citric + γ-Aminobutyric	1.12	1.01	0.98

[1]($K_{amino\ acid\text{-}citrate}/K_{citrate}$)

described as possessing dual functions, namely, the amino acid serves as an amine source and as acid catalyst in the reaction with sugars (Shallenberger and Birch 1975). When we consider the acidity of natural grapefruit juice, as well as the acidity in our simulated model systems, the basic amino group may not be available as an amine source to interact with the sugar carbonyl group. Any catalytic action of amino acids is probably due to its contribution to the total ionic strength, or to the hydrogen ion activity of the reaction system.

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CARBOHYDRATE UTILIZATION AND GROWTH KINETICS IN THE PRODUCTION OF YOGURT FROM SOYMILK. PART II: EXPERIMENTAL AND PARAMETER ESTIMATION RESULTS[1]

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ABSTRACT

Typical yogurt bacteria, Lactobacillus bulgaricus *and* Streptococcus thermophilus, *have been studied for their ability to utilize stachyose and other sugars in soymilk. Subculturing of the inocula on nonfat dry milk powder (NFDM) yielded bacteria that displayed oligosaccharide formation during fermentation of soymilk. However, storage of stock cultures on soymilk for 168 h or longer resulted in stachyose hydrolysis during fermentation. Values of the maximum specific growth rate for mixed cultures of* Lactobacillus bulgaricus *and* Streptococcus thermophilus *grown on soymilk at 44°C were estimated using the covariate adjustment method which allows biomass, substrate, and product data to be employed, simultaneously. Point estimates were 0.341, 0.296, and 0.478* h^{-1} *for soymilk fermentation by inocula subcultured on 11% NFDM, for inocula stored in soymilk for greater than 168 h, and for inocula prepared as a mixed culture, respectively. The available electron balance (A.E.B.) was employed to check the consistency of the data using biomass, substrate and product measurements.*

INTRODUCTION

For soymilk-based products to become popular for human consumption in the United States, the objectional beany flavor typically associated with soya products and the flatulence factor associated with raffinose and stachyose should be re-

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duced or eliminated. Buono (1988) addresses beany flavor in organoleptic studies conducted on soymilk yogurts. In the present work, microbial utilization of stachyose in soymilk is examined.

Yogurt is made by fermentation of milk using lactic acid bacteria. The classical yogurt bacteria are *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Studies of stachyose reduction during fermentation of soymilk have been reported in the literature (Mital and Steinkraus 1975; Pinthong *et al.* 1980). Most studies have focused on the following lactic acid bacteria: *L. fermenti, L. fermentum, L. plantarum,* and *L. cellobiosis* and the ability of pure and mixed cultures to consume stachyose in soymilk. In the present work, the focus is on stachyose consumption by commonly used yogurt bacteria, i.e., *L. bulgaricus* and *S. thermophilus* in mixed culture. Consideration is given to method of inoculum preparation, type of milk in which the stock cultures are stored, and length of storage time.

Industrial design and scale-up of fermentation require accurate information on the kinetic and yield parameters that define microbial growth and product formation. As much information as possible should be incorporated into the parameter estimation process. Further, using all available measurements permits the closure of the mass and available electron balances to be evaluated. This allows detection of possible measurement errors and can even suggest alternate pathways of metabolic activity of the bacteria studied.

The parameter estimation method employed to calculate point estimates for the maximum specific growth rate of yogurt bacteria is the one developed by Oner *et al.* (1986). This method uses biomass, substrate, and product data to select the region of exponential growth. Further, the maximum specific growth rate is estimated using all of the data and the covariate adjustment method (Erickson *et al.* 1988).

MATERIALS AND METHODS

Procedures for measurement of microbial population, lactic acid concentration, and sugar concentrations are discussed by Buono (1988). Point estimates of the maximum specific growth rate and 95% confidence intervals were made by regression analysis using the Statistical Analysis System (SAS) software package (Oner *et al.* 1986; Erickson *et al.* 1988).

Inoculum preparation followed the technique outlined by Buono (1988). A 5 mL vial of the pure culture was used to inoculate each first round-flask; these subcultures were incubated for 24 h at 44°C. Second-round flasks were inoculated with a 5% by volume inoculum and incubated for 12 h at 44°C. Third-round flasks were inoculated with a 5% by volume inoculum, incubated for 3 h at 44°C, and then used as the inoculum for the experiment. The stock cultures were

stored in the freezer and were regenerated monthly. The method of using four or five test tubes to prepare a physically blended mixed culture inoculum from pure cultures, for inoculation into fourth-round flask(s), is described in Part I (Buono *et al.* 1990) and by Buono (1988). However, the type of milk on which the organisms were stored and into which they were inoculated was varied in this work (see Table 1). Study I used pure culture, 5 mL vials of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* stored on 11% NFDM with 17% sterile glycerol at −10°C in an International Harvester Model 70 freezer. Organisms in study I were subcultured on 11% NFDM from first-round to second-round flasks. The third-round of flasks contained sterile soymilk. These third-round, pure cultures were blended physically to obtain a mixed culture and were used to inoculate fourth-round flasks, which also contained sterile soymilk. All sampling was from the fourth-round flasks.

Studies II, III, and IV used pure culture, 5 mL vials of the bacteria stored at −10°C on soymilk plus 17% glycerol. In addition, all subculturing was in soymilk. The stock cultures were stored on soymilk for different lengths of time: 12, 168, and 336 h, respectively, for studies II, III, and IV. All inocula were subcultured as pure cultures up to the third round of flasks. The fourth-round

TABLE 1.
MEDIA AND CULTURES OF STUDIES IA THROUGH VI

	Study Code						
	IA	IB	II	III	IV	V	VI
Storage Milk*	NFDM p	NFDM p	SOY p	SOY p	SOY p	NFDM p	SOY p
First-Round Flask	NFDM p	NFDM p	SOY p	SOY p	SOY p	NFDM p	SOY p
Second-Round Flask	NFDM p	NFDM p	SOY p	SOY p	SOY p	NFDM m	SOY m
Third-Round Flask	SOY p	SOY p	SOY p	SOY p	SOY p	SOY m	SOY m
Fourth-Round Flask	SOY m	SOY m	SOY m	SOY m	SOY m	SOY m	SOY m
Storage Time on Soymilk, Hours	0	0	12	168	336	0	168

* NFDM: Nonfat dry milk
* SOY: Soymilk
* p: Pure Culture
* m: Mixed Culture

flasks were inoculated with a physically blended mixed culture of the two pure cultures.

Studies V and VI considered preparation of the inocula by subculturing as a mixed culture rather than being physically blended. Two 250 mL flasks containing 100 mL of milk were inoculated using the pure culture 5 mL vials out of the freezer. These first-round flasks were incubated for 24 h at 44°C. One second-round flask was inoculated with 5% by volume of fermented milk from the first-round flask that contained *Streptococcus thermophilus*. This single-second round flask was incubated along with the other first-round flask. After 6 h, 5% by volume from the first-round flask, which contained *Lactobacillus bulgaricus,* was used to inoculate the single second-round flask, which contained the 6-hour-old culture of *Streptococcus*. The mixed culture in the second-round flask was incubated for 6 h. A single third-round flask was inoculated with 5% by volume of the mixed culture of lactic acid bacteria. The third-round flask was incubated for 3 h, after which it served as inoculum for the fourth-round flask. All sampling was made from the fourth round flask. Study V used 11% NFDM plus glycerol in storage and NFDM in first- and second-round flasks. In study VI, soymilk was used in storage and in all rounds of the inoculum train.

Two fermentations were conducted in study I (IA and IB) to examine precision of the work. Both fermentations IA and IB, were considered in the estimation of maximum specific growth rate whereas only study IB was considered in the determination of stachyose utilization. The rationale for this use of the data was based upon the fact that fermentation IA was analyzed by HPLC for sugar concentration prior to selecting the 70:30 acetonitrile:water mobile phase (Buono 1988).

Table 1 summarizes the differences between studies IA, IB, II, III, IV, V, and VI.

THEORY

The carbon mass balance during fermentation is

$$\text{CONSUMED SUBSTRATE} = \text{GROWTH} + \text{LACTIC ACID FORMATION} + CO_2 \quad (1)$$

Sucrose and stachyose are considered as the substrates used for growth and product formation based upon experimental evidence. Since lactic acid bacteria are known homofermenters, CO_2 production is small. Thus, it is desirable to utilize the available electron balance and the reductance degree, γ, which is the number of equivalents of available electrons per gram mole carbon (Erickson *et al.* 1978). A physiological dead state is used with the valences C = 4, H = 1,

O = −2, and N = −3. Under anaerobic conditions, the available electron balance in integrated form is (Oner *et al.* 1986)

$$\frac{\left\{ \frac{\gamma_b \sigma_b}{12}(X - X_o) + \frac{\gamma_p \sigma_p}{12}(P - P_o) \right\}}{\left[\frac{[(S_{suo} - S_{su})\sigma_{su} + (S_{sto} - S_{st})\sigma_{st}]\gamma_s}{12} \right]} = 1 \tag{2}$$

A simple statement of the same balance is realized by introduction of biomass energetic yield, η, and product energetic yield, ξ, into Eq. (2), i.e.,

$$1.0 = \eta + \xi \tag{3}$$

where

$$\eta = \frac{\frac{\sigma_b \gamma_b}{12}(X - X_o)}{\left[\frac{[(S_{suo} - S_{su})\sigma_{su} + (S_{sto} - S_{st})\sigma_{st}]\gamma_s}{12} \right]} \tag{4}$$

$$\xi = \frac{\frac{\sigma_p \gamma_p}{12}(P - P_o)}{\left[\frac{[(S_{suo} - S_{su})\sigma_{su} + (S_{sto} - S_{st})\sigma_{st}]\gamma_s}{12} \right]} \tag{5}$$

Equation (2) or (3) is used to check data consistency.

The maximum specific growth rate in a set cup yogurt reactor under exponential growth conditions is assumed to be a constant given by the equation

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{6}$$

or

$$\mu = \mu_{max} = \text{constant} \tag{7}$$

Using a constant yield, substrate and product measurements were converted into biomass equivalents. Substrate biomass equivalents are defined by Z, e.g.,

$$Z = \frac{X_o \sigma_b \gamma_b}{\gamma_s \eta_1} + [\sigma_{su}(S_{suo} - S_{su}) + \sigma_{st}(S_{sto} - S_{st})] \tag{8}$$

where X_o, S_{suo}, and S_{sto} are the biomass concentration, sucrose concentration, and stachyose concentration at the start of the exponential growth interval, respectively. Similarly, for product data, lactic acid biomass equivalents are expressed in terms of Y, e.g.,

$$Y = \frac{X_o \sigma_b \gamma_b \xi_1}{\sigma_p \gamma_p \eta_1} + (P - P_o) \tag{9}$$

where P_o is the concentration of lactic acid measured at the start of the exponential growth interval, and σ_p and γ_p are the weight fraction of carbon in lactic acid and the reductance degree of lactic acid, respectively. In addition, during exponential growth, the biomass energetic yield, η, and the product energetic yield, ξ, are assumed constant, i.e.,

$$\eta = \eta_1 \tag{10}$$

$$\xi = \xi_1 \tag{11}$$

where η_1 and ξ_1 are the constant values for the biomass and product energetic yields in Eq. (8) and (9).

Since Z and Y represent biomass equivalent measurements, μ in Eq. (6) can be written alternately as (Oner *et al.* 1986).

$$\mu = \frac{1}{Z}\frac{dZ}{dt} \tag{12}$$

$$\mu = \frac{1}{Y}\frac{dY}{dt} \tag{13}$$

To determine the maximum specific growth rate, Eq. (6) is integrated from the start of the exponential growth interval, t_o, to any point of the exponential growth interval, t, viz.,

$$\ln X = \mu_{max}(t - t_o) + \ln X_o \tag{14}$$

Similar manipulation of Eq. (12) and (13) gives two additional equations of the form ln Z and ln Y versus t, viz.,

$$\ln Z = \mu_{max}(t - t_o) + \ln Z_o \tag{15}$$

$$\ln Y = \mu_{max}(t - t_o) + \ln Y_o \tag{16}$$

A plot of ln X, ln Z, or ln Y versus time should give a straight line with slope equal to μ_{max} in the exponential growth interval. Slope estimates obtained from the regressions are referred to as point estimates of μ_{max}. The point estimates from Eq. (14), (15) and (16) are determined using biomass, substrate, and product data, respectively. By combining these data and by using the covariate adjustment technique, estimates based on the substrate, biomass, and product data can be obtained (Erickson *et al.* 1988).

A prerequisite for estimation of μ_{max} using covariate adjustment is knowledge of the interval of exponential growth. A rough estimate of this exponential growth interval can be made from the raw data by a visual examination of a plot of ln

X versus time. The portion of the plot that best resembles a straight line, as determined by an eye fit, serves as the first approximation of the interval of exponential growth. SAS can be used to fit regressions of ln X, ln Z, and ln Y to the eye-selected, exponential growth interval as follows,

$$\ln X = A_1 + B_1 t + C_1 t^2 \tag{17}$$

$$\ln Z = A_2 + B_2 t + C_2 t^2 \tag{18}$$

$$\ln Y = A_3 + B_3 t + C_3 t^2 \tag{19}$$

Selection of the time interval involves adding one data point at a time to the initial eye-fit interval. Values of T, $\sqrt{\text{MSE}}$, and R for the new time interval are calculated. T indicates the degree of significance of the quadratic term (significance for $T \leq 0.1$), $\sqrt{\text{MSE}}$ is the mean square error of the specific growth rate estimate, and R is the residual calculated for the added data point by a least squares fit to the data. The criteria used to make the final selection of the exponential interval are: a value for $T \geq 0.1$ indicating that the quadratic term is negligible; a small value for $\sqrt{\text{MSE}}$ of the estimate; and a small value for the residual of the added data point, R.

To calculate values for Z and Y, i.e., to convert the substrate and product data into biomass equivalents by Eq. (8) and (9), respectively, estimates for η_1 and ξ_1 are needed. These estimates may be obtained using the methods of Oner *et al.* (1986), which involve Eq. (4) and (5), the experimental data, and linear regression. Values of η_1 and ξ_1 can be calculated from the slopes of the linear regressions. To initiate calculations for selection of the exponential growth interval, the data fit by eye to the ln X versus time plot are used as a first approximation of the exponential growth interval. This interval is used in the regression analysis of Eq. (4) and (5) to obtain first-round approximations for η_1 and ξ_1. These yield estimates are used in Eq. (8) and (9) to generate values for Z and Y, respectively. Using statistical analysis, the exponential growth interval is selected. New estimates are made for η_1 and ξ_1 in the best, selected, exponential interval. Again, estimates for the yield quantities are made by a linear regression on Eq. (4) and (5). The methods are similar to those used by Oner *et al.* (1986) and are described in detail in Buono (1988).

RESULTS AND DISCUSSION

In Fig. 1, biomass, lactic acid, stachyose, and sucrose concentration are plotted versus time for Study III. Similar data were collected for each experiment (Buono 1988). Cell number of each species, as measured by direct microscopic count of rods and cocci, were also tabulated (Buono 1988). Sucrose appeared to be the main source of carbon and energy for growth and lactic acid formation.

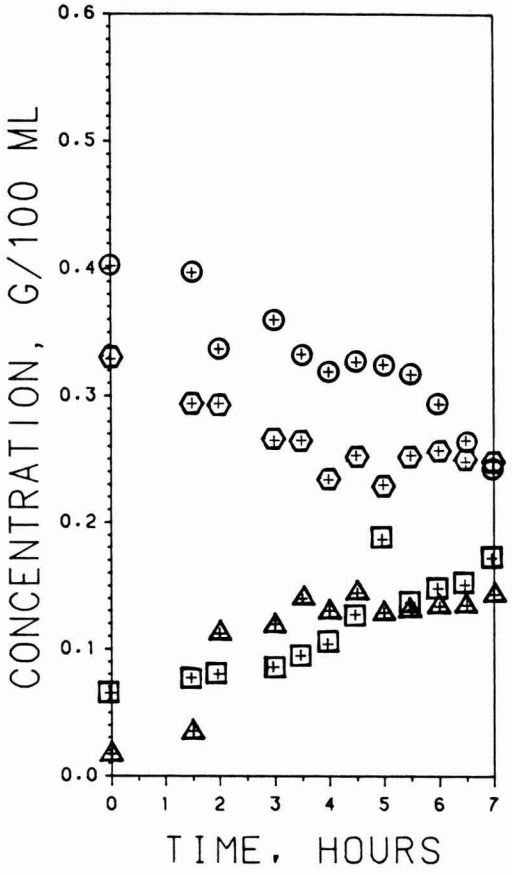

FIG. 1. CONCENTRATIONS OF SUCROSE (○), STACHYOSE (⬡), BIOMASS (△), AND LACTIC ACID (□) PLOTTED VERSUS TIME FOR STUDY III

Sucrose consumption ranged from 40% in Study III to 86% in Study V. Stachyose concentration in Study III decreased from 0.329 g/100 mL to 0.248 g/100 mL during the fermentation (Fig. 1). In Fig. 2-7 the concentration of stachyose and total viable cell counts are plotted versus time for studies IB, II, III, IV, V, and VI, respectively. In Fig. 8 and 9, pH and titratable acidity also are presented as a function of time.

Oligosaccharide Formation and Reduction of Stachyose

In Study IB the stachyose concentration increases from 0.239 to 0.357 g/100 mL as the fermentation proceeds from start to finish (Fig. 2). The organisms in study IB were stored on 11% NFDM and grown in first- and second-round flasks

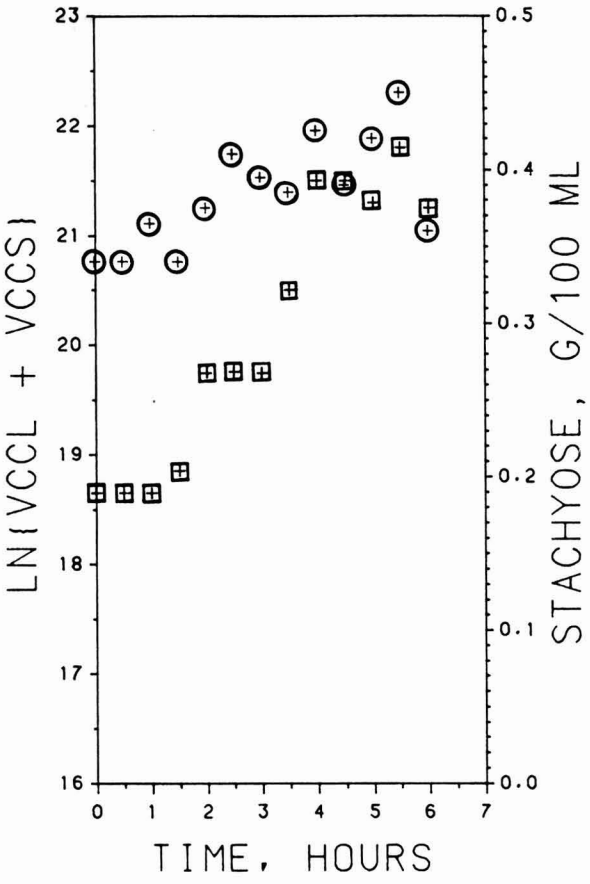

FIG. 2. DEMONSTRATION OF OLIGOSACCHARIDE FORMATION BY A MIXED CULTURE OF *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* GROWN ON SOYMILK

Results were taken from study IB, in which the inocula were stored and propagated on 11% nonfat dry milk. Stachyose concentration,O, and the sum of the viable cell count of *Lactobacillus*, VCCL, and *Streptococcus*, VCCS, □, are plotted versus time.

on 11% NFDM (see Table 1). For the same time interval of the fermentation in which stachyose concentration was observed to increase, the concentration of lactic acid also increased but sucrose concentration decreased. The observed increase in stachyose concentration arising from fermentation was due to oligosaccharide formation (Prenosil *et. al.* 1987). Basically, oligosaccharide formation results from the transfer activity of galactosidase enzymes produced by lactic acid bacteria.

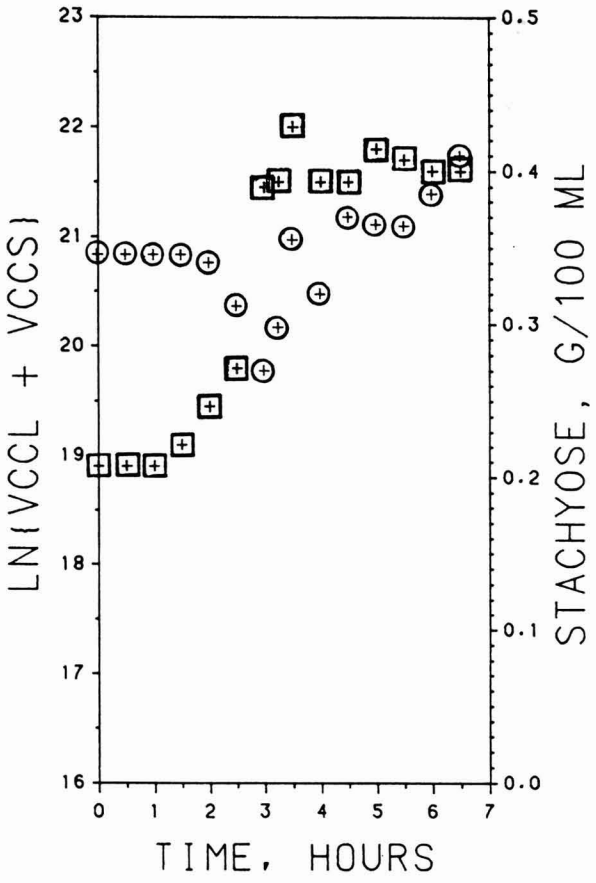

FIG. 3. DEMONSTRATION OF POSSIBLE INDUCED STACHYOSE UTILIZATION FOLLOWED BY OLIGOSACCHARIDE FORMATION IN A MIXED CULTURE OF *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* GROWN ON SOYMILK

Results were taken from study II in which the inocula were stored for 12 h on soymilk and also propagated on soymilk prior to inoculation. Stachyose concentration, O, and the sum of the viable cell count of *Lactobacillus*, VCCL, and *Streptococcus*, VCCS, □, are plotted versus time.

Study II data plotted in Fig. 3 show a reduction in the stachyose concentration for the time period 0.0 to 3.25 h from 0.330 to 0.270 g/100 mL. After hour 4.0, the concentration of stachyose increased from 0.320 to 0.393 g/100 mL. This increase was attributed to oligosaccharide formation. However, the differences in the shape of the stachyose curves in Fig. 2 through 5, either positively or negatively sloped, require additional discussion. The decrease in stachyose concentration in studies III and IV was due to hydrolysis. The primary difference

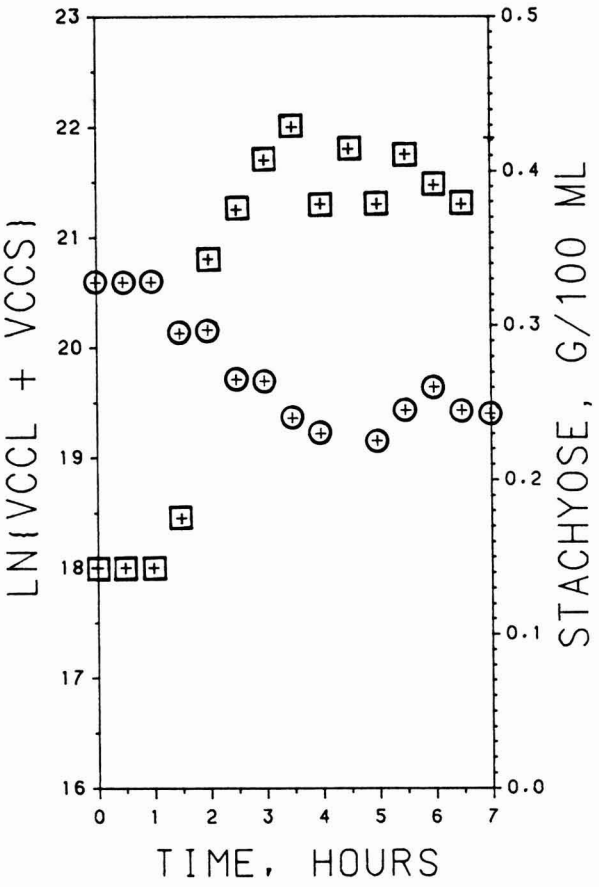

FIG. 4. CONSUMPTION OF STACHYOSE RECORDED FOR A MIXED CULTURE OF *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* GROWN ON SOYMILK

Results were taken from study III, in which the inocula were stored for 168 hours on soymilk and also propagated on soymilk prior to inoculation. Stachyose concentration, O, and the sum of the viable cell count of *Lactobacillus*, VCCL, and *Streptococcus*, VCCS, □, are plotted versus time.

between study IB and studies II, III and IV was in the milk in which the inoculum was stored and incubated (see Table 1). In studies II, III, and IV, the inoculum was stored and incubated in soymilk. The storage time in soymilk prior to first round incubation was 12 h for study II, 168 h for study III, and 336 h for study IV. Storage time seems to play the largest role in reduction of stachyose concentration. When the bacteria were stored on soymilk at $-10°C$ for greater than 168 h, an obvious reduction in stachyose concentration was observed in the soymilk fermentations. Net decreases in stachyose concentration of 0.081 and

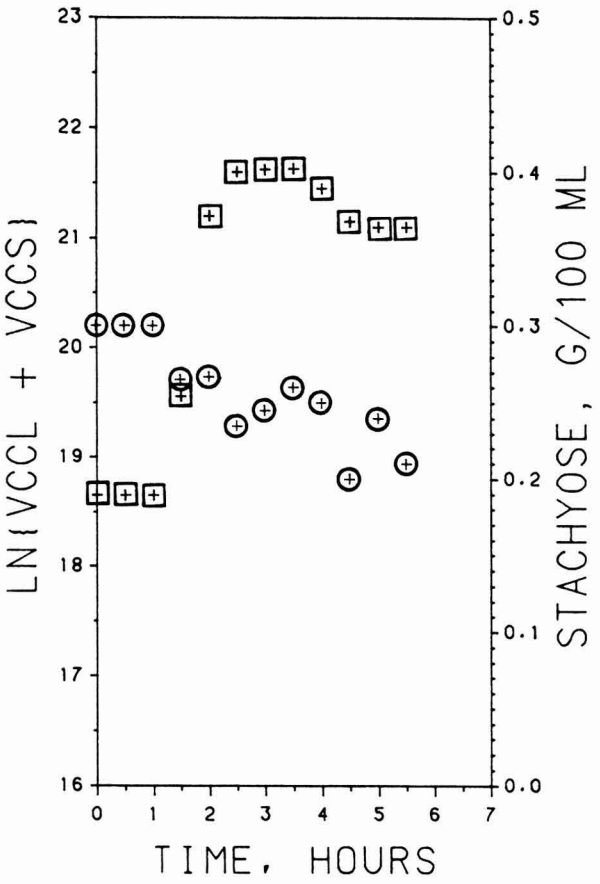

FIG. 5. CONSUMPTION OF STACHYOSE RECORDED FOR A MIXED CULTURE OF *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS* GROWN ON SOYMILK

Results were taken from study IV, in which the inocula were stored for 336 hours on soymilk and also propagated on soymilk prior to inoculation. Stachyose concentration, O, and the sum of the viable cell count of *Lactobacillus*, VCCL, and *Streptococcus*, VCCS, □, are plotted versus time.

0.083 g/100 mL, respectively, were recorded for Studies III and IV. Surprisingly, for the bacteria in study II there was a net increase in the stachyose concentration from 0.330 to 0.393 g/100 mL. The minimum in the stachyose curve observed at 3.25 h in study II was not observed in study IB. The minimum together with the smaller increase in stachyose concentration suggests that the increase probably resulted from the galactosidase enzyme functioning in a transfer reaction rather than in a hydrolysis reaction, i.e., oligosaccharide formation. It is possible that the water and carbohydrates in the soymilk were in competition at the functional

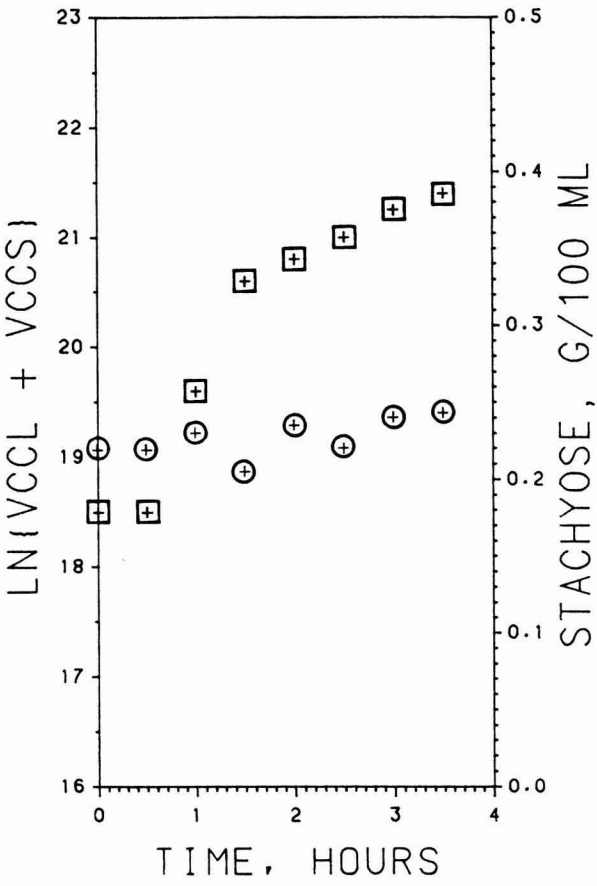

FIG. 6. CONCENTRATION PROFILE OF STACHYOSE ON SOYMILK AS A RESULT OF A MIXED CULTURE FERMENTATION USING *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS*

Results were taken from study V, in which the inoculum was prepared as a mixed culture prior to inoculation. Stachyose concentration, O, and the sum of the viable cell count of *Lactobacillus*, VCCL, and *Streptococcus*, VCCS, □, are plotted versus time.

site on the galactosidase enzyme. The key point of the competitive nature of the active site of galactosidase is that exposure to soymilk for more than 168 h facilitated stachyose consumption.

Also of interest in all of these figures was total time of the fermentation. The maximum length of time for any of the soymilk fermentations was 7 h. The fermentation was continued until the pH of the milk had reached about 4.5, the average pH value reported for yogurt.

In an effort to make yogurt production from soymilk by fermentation economically desirable, the method of inoculum preparation was varied to try to

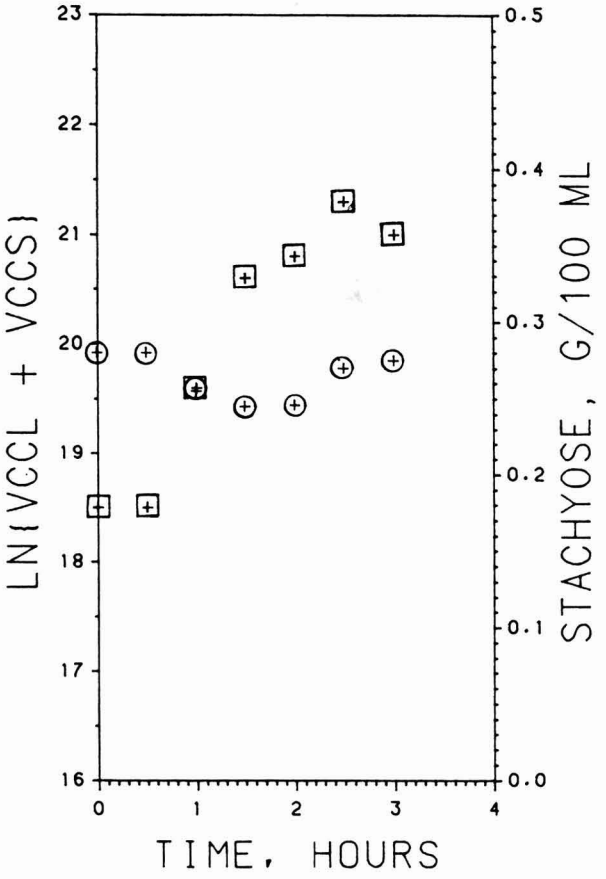

FIG. 7. CONCENTRATION PROFILE OF STACHYOSE ON SOYMILK AS A RESULT OF A MIXED CULTURE FERMENTATION USING *LACTOBACILLUS BULGARICUS* AND *STREPTOCOCCUS THERMOPHILUS*
Results were taken from study VI, in which the inoculum was prepared as a mixed culture prior to inoculation. Stachyose concentration, O, and the sum of the viable cell count of *Lactobacillus*, VCCL, and *Streptococcus*, VCCS, □, are plotted versus time.

bring the time of fermentation to 3–4 h, which is current practice in the yogurt business. As shown in Table 1, in studies V and VI, the inocula were prepared as a growing mixed culture of bacteria rather than as pure cultures that had been physically mixed. Figures 6 and 7 show the growth of these bacteria in soymilk and the stachyose profiles. Study V was similar to study IB in that the bacteria were stored on 11% NFDM and treated in first- and second-round flasks with 11% NFDM. The third round flask contained soymilk. Study VI was similar to study III in that the bacteria were stored in soymilk at −10°C for 168 h. The

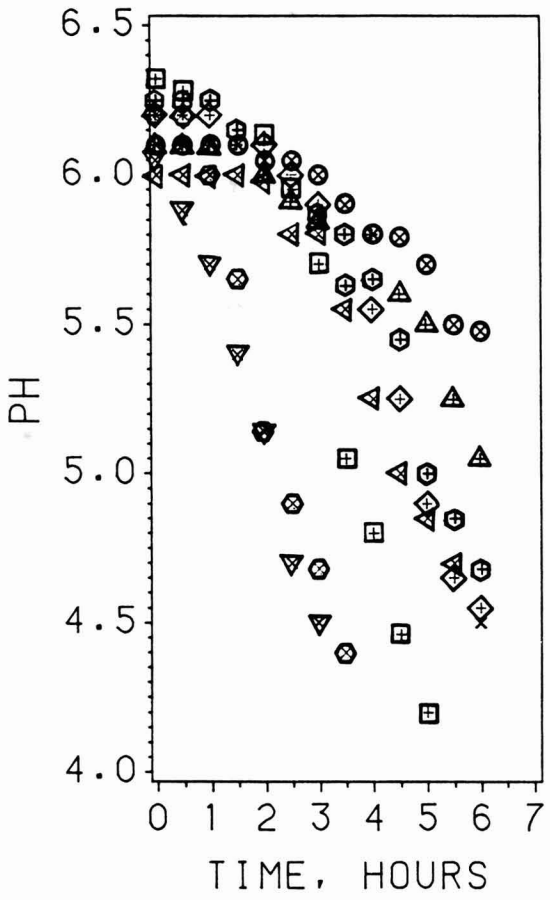

FIG. 8. pH PROFILES OF MIXED CULTURE FERMENTATIONS OF EITHER SOYMILK OR FORTIFIED BOVINE MILK USING *L. BULGARICUS* AND *S. THERMOPHILUS* AS THE MIXED CULTURE

□ represents fortified bovine milk fermentation; ⬡ represents results of study IA; ◇ represents results of study IB; △ represents results of study II; ○ represents results of study III; ◁ represents results of study IV; ⬡ represents results of study V; ▽ represents results of study VI (See Table 1).

desired pH of 4.5 was reached approximately 30 min earlier in study VI than in study V. There was also some evidence of the hypothesized enzyme induction in the stachyose profiles for the two fermentations. Study VI displayed a dip in the stachyose concentration not unlike that observed in study II. However, in terms of overall reduction of stachyose, the mixed culture inocula did not approach the pure culture inocula. There was a significant reduction in the total time required to reach pH 4.5 in both fermentations.

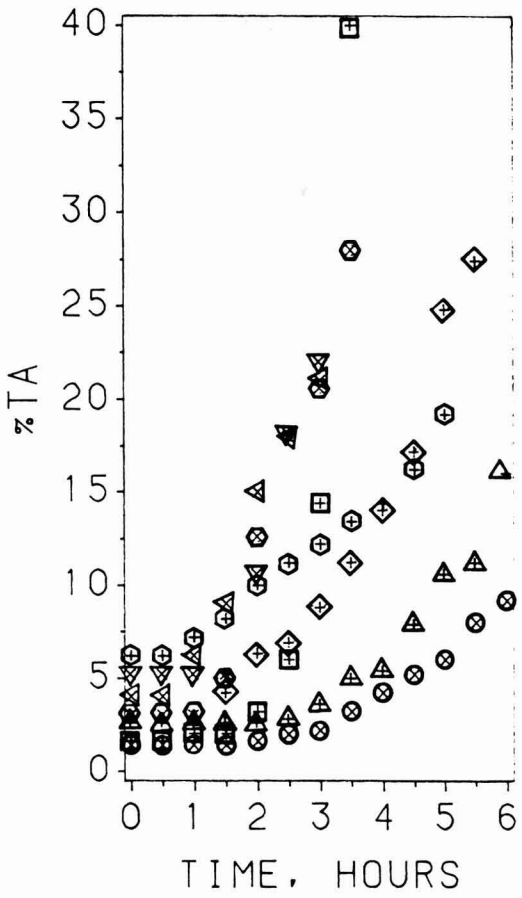

FIG. 9. PERCENT TITRATABLE ACIDITY OF MIXED CULTURE FERMENTATIONS OF EITHER SOYMILK OR FORTIFIED BOVINE MILK

□ represents fortified bovine milk fermentation; ⬡ represents results of study IA; ◇ represents results of study IB; △ represents results of study II; ○ represents results of study III; ◁ represents results of study IV; ⬡ represents results of study V; ▽ represents results of study VI. % TA was expressed as the volume (mL) of 0.1 N NaOH titrant added per mL of sample times 100.

In studies V and VI, 86% and 80%, respectively, of the sucrose was consumed. This compares well with the sucrose used in the other studies. The maximum stachyose consumption occurred in study IV, in which 27% of the stachyose was hydrolyzed. Based on sucrose consumption and lactic acid production, the mixed culture inoculum realized a low pH in about half the fermentation time required when using a pure culture inoculum. However, the longer, slower fermentation was more desirable in bringing about a reduction in stachyose.

Selection of the Interval of Exponential Growth and Estimation of μ_{max}

Estimates of the maximum specific growth for studies IA, IB, III, and V were made using biomass, substrate, and product data. To determine a first guess estimate of the exponential growth interval, the natural log of the biomass concentration was plotted against time for each data set. The data were examined to locate the region that was closest to a perfectly straight line, or the first guess of the region of exponential growth. The intervals selected are listed in Table 2. Together with first approximations to η_1 and ξ_1 obtained from the data, as discussed in the theory section above, the estimates for the yields were inserted into Eq. (8) and (9) to obtain Z and Y, respectively. Equations (14), (15), and (16) were used to estimate the intervals of exponential growth for the fermentations. Table 3 summarizes the T value, $\sqrt{MSE}$, and residual of the added data point, R, obtained using substrate, biomass, and product data for all data points in each of the four fermentations. The asterisks indicate the intervals selected as the exponential growth interval.

The rationale employed for each data set was the same. Selection of the exponential growth interval for soymilk IA is discussed below to illustrate the criteria involved in the selection process. The biomass data, substrate data, and the product data are presented in Table 3. RX, RZ, and RY corresponded to the residuals of the added data point for the biomass, substrate and product data, respectively. The added data point in each interval is underlined. Examination of RX, RZ, and RY values for soymilk IA in Table 3 shows that the smallest value occurs for interval 1.0–4.5 for biomass and substrate data and for 1.0–5.0 for product data. Small T values indicate the significance of the quadratic term in Eq. (24), (25), and (26); that is, $T \leq 0.1$. For soymilk IA, the largest value of T for biomass, substrate, and product data was for intervals 1.0–5.0, 1.0–4.0, and 0.0–4.0, respectively. Thus, the interval 1.0–5.0 had satisfied two criteria, i.e., smallest residual and largest value of T. Finally, $\sqrt{MSE}$ of the

TABLE 2.
EYE FIT EXPONENTIAL GROWTH INTERVALS AND FIRST ESTIMATES OF η_1 AND ξ_1 FOR FOUR SOYMILK FERMENTATIONS

Study	η_1	ξ_1	Expon. Growth Interval
IA	0.791	0.481	1.0 - 4.0 h
IB	0.653	0.404	1.0 - 4.0 h
III	0.428	0.208	1.5 - 3.5 h
V	0.449	0.820	0.0 - 3.0 h

TABLE 3.
T VALUES, $\sqrt{MSE}$, AND RESIDUALS USED TO SELECT EXPONENTIAL GROWTH INTERVALS FOR STUDIES IA, IB, III, AND V

	Biomass			Substrate			Product		
Interval	$\sqrt{MSE}$	T	RX	$\sqrt{MSE}$	T	RZ	$\sqrt{MSE}$	T	RY
Study IA									
1.-4.	.223	.099	-	.377	.109	-	.140	.2	-
0.-4.	.204	.224	.071	.548	.008	.673	.167	.531	.173
1.-4.5	.199	.234	.009	.373	.053	.274	.147	.064	.133
*1.-5.	.187	.512	.085	.347	.082	.479	.142	.486	.085
Study IB									
1.-4.	.159	.822	--	.295	.011	-	.174	.022	-
1.-5.	.194	.307	.191	.291	.039	.176	.167	.062	.089
*1.-5.5	.202	.118	.176	.296	.014	.239	.154	.218	.034
1.-6.	.226	.028	.267	.291	.008	.207	.145	.17	.061
Study III									
1.5-3.5	.635	.925	-	.081	.302	-	.059	.454	-
0.-3.5	.538	.714	.128	.101	.415	.068	.148	.229	.125
1.5-4.5	.451	.836	.061	.111	.529	.106	.061	.105	.034
*1.5-5.	.422	.566	.211	.109	.276	.076	.056	.441	.018
Study V									
*0.-3.	.139	.192	-	.201	.798	-	.093	.092	-
0.-3.5	.161	.029	.133	.180	.522	.085	.079	.08	.028

Underlined values are the added data points.
* Selected time interval.

estimate was smallest for the biomass, substrate, and product data for the intervals 1.0–5.0, 1.0–5.0, and 0.0–4.0, respectively. This final test gives four criteria satisfied out of a possible nine for the interval 1.0–5.0. This is the largest number of criteria satisfied for any interval. Consequently, the interval 1.0–5.0 was selected as the exponential growth interval for soymilk IA. Similar testing of the data was carried out for studies IB, III, and V.

Table 4 lists the selected, exponential, growth intervals for the four fermentations considered along with revised estimates for the exponential yield parameters, η_1 and ξ_1. Revised yield estimates were made using the newly selected, exponential intervals, as described in the theory section above. Values for Z and Y were calculated with X_o, P_o, S_{suo} and S_{sto} set as the values for these variables

TABLE 4.
STATISTICALLY SELECTED INTERVALS OF EXPONENTIAL GROWTH AND REVISED ESTIMATES FOR EXPONENTIAL YIELDS η_1 AND ξ_1

Study	Statistically selected exponential interval	η_1	ξ_1
IA	1.0 - 5.0	0.484	0.388
IB	1.0 - 5.5	0.525	0.609
III	1.5 - 5.0	0.562	0.256
V	0.0 - 3.0	0.488	0.763

at the start of the newly selected exponential growth intervals. Ln X, ln Z and ln Y then were fit to a linear regression by least squares analysis on time to estimate the slope, μ_{max}. Tables 5 through 8 list values of the point estimates and 95% confidence intervals using biomass, substrate, and product data, for studies IA, IB, III, and V, respectively. The estimate using all data, i.e., biomass, substrate, and product data, and having the narrowest width of the 95% C.I. was chosen as the best estimate. For studies IA, IB, III, and V, these estimates were 0.341, 0.373, 0.296, and 0.478 hour,$^{-1}$ respectively. Clearly, soymilk V,

TABLE 5.
POINT AND 95% CONFIDENCE INTERVAL ESTIMATES OF μ_{max} FOR STUDY IA

Data	Pt Est.	Cov.	95% C.I.	Width 95% C.I.
X	0.390	NONE	[0.265, 0.515]	0.251
Z	0.276	NONE	[0.128, 0.424]	0.296
Y	0.351	NONE	[0.276, 0.426]	0.150
X, Z, Y	0.341*	NONE	[0.300, 0.383]	0.084
X, Z, Y	0.342	Z_1	[0.105, 0.579]	0.474
X, Z, Y	0.360	Z_2	[0.277, 0.443]	0.166
X, Z, Y	0.360	Z_1, Z_2	[0.261, 0.459]	0.197

* Result with smallest 95 % confidence interval based on all data.

TABLE 6.
POINT AND 95% CONFIDENCE INTERVAL ESTIMATES OF μ_{max} FOR STUDY IB

Data	Pt Est	Cov	95% C.I.	Width 95% C.I.
X	0.363	NONE	[0.243, 0.483]	0.240
Z	0.327	NONE	[0.174, 0.479]	0.304
Y	0.366	NONE	[0.312, 0.421]	0.110
X, Z, Y	0.352	NONE	[0.267, 0.437]	0.170
X, Z, Y	0.353	Z_1	[0.263, 0.442]	0.179
X, Z, Y	0.372	Z_2	[0.298, 0.445]	0.147
X, Z, Y	0.373*	Z_1, Z_2	[0.308, 0.439]	0.139

* Result with smallest 95 % confidence interval based on all data.

TABLE 7.
POINT AND 95% CONFIDENCE INTERVAL OF μ_{max} ESTIMATES FOR STUDY III

Data	Pt Est.	Cov.	95% C.I.	Width 95% C.I.
X	0.308	NONE	[-.065, 0.681]	0.746
Z	0.304	NONE	[0.189, 0.418]	0.229
Y	0.253	NONE	[0.099, 0.407]	0.308
X, Z, Y	.289	NONE	[0.173, 0.404]	0.231
X, Z, Y	.314	Z_1	[0.129, 0.498]	0.368
X, Z, Y	.296*	Z_2	[0.182, 0.411]	0.229
X, Z, Y	.306	Z_1, Z_2	[0.075, 0.537]	0.461

* Result with smallest 95 % confidence interval based on all data.

TABLE 8.
POINT AND 95% CONFIDENCE INTERVAL OF μ_{max} ESTIMATES FOR STUDY V.

Data	Pt Est.	Cov.	95% C.I.	Width 95% C.I.
X	0.475	NONE	[-.340, 1.29]	1.63
Z	0.478	NONE	[-.767, 1.72]	2.49
Y	0.483	NONE	[-.034, 1.00]	1.03
X, Z, Y	0.479	NONE	[-.073, 1.03]	1.10
X, Z, Y	0.473	Z_1	[-.186, 1.13]	1.32
X, Z, Y	0.478*	Z_2	[0.474, 0.481]	0.006

* Result with smallest 95 % confidence interval based on all data.

which was fermented using a mixed culture inoculum, produced an accelerated rate of bacterial growth. This result was indicated by the length of time necessary to reach a pH of 4.5. As discussed above, only 3.0 hours were required to reach the final pH for study V compared to 6 or 7 h for the other soymilk fermentations. This fact is reflected in the larger value estimated for μ_{max}.

The estimated values of μ_{max} are in good agreement with the values reported by Oner *et al.* (1986) for *Lactobacillus bulgaricus* and *Streptococcus thermophilus* growing on nonfat dry milk. Oner *et al.* (1986) reported values ranging from 0.28 h^{-1} to 0.47 h^{-1}; for mixed cultures, they estimated that μ_{max} = 0.41 h^{-1}.

Available Electron Balance and Data Consistency

A check on the accuracy of the yield parameter estimates was conducted by calculation of the available electron balance, Eq. (3). From Eq. (3), the sum of η_1 and ξ_1 in Table 4 should equal 1.00 for perfect estimates made from perfect experimental data. In actual experimental studies, measurement error is always present. The error filters through the parameter estimation procedures into the estimates themselves. For studies IA, IB, III, and V the available electron balances calculated from the yield estimates were 0.87, 1.13, 0.82, and 1.25, respectively, all reasonably close to 1.00.

Examination of the Available Electron Balance and Further Discussion of η and ξ

The consistency based on the initial and final measurements is presented in Table 9. A check on the consistency of the entire data set, i.e., biomass, substrate and product measurements, also was made using Eq. (3). Values of the available electron balance determined for studies IB, II, III, IV, V, and VI as a function of time are presented in Buono (1988). Study IB and II, in which the bacteria generated oligosaccharides during the fermentation, had many values of the available electron balance that were greater than 1.0. This behavior suggested that more biomass and lactic acid were being produced than could be accounted for by the measured substrate consumption. There were substrate sources other than sugars that could be consumed by the bacteria. Babcock fat tests conducted on several soymilk fermentations showed that the fat in soymilk was reduced after 6 h of fermentation. In the studies in which the bacteria appeared to reduce the stachyose concentration, i.e., studies III and IV, there was good closure of the available electron balance compared to the other studies.

Studies V and VI, in which the inocula were grown as a mixed culture, showed similar results. The values of the available electron balance were greater than 1.0. In Table 9, the data consistency is better for study VI than for study V. Moreover, Fig. 6 and 7 show that stachyose utilization is better in study VI.

Additional insight into the microbiological processes that occur during soymilk fermentation can be obtained by examination of the biomass yield, η, for anaerobic growth. Oner *et al.* (1984) have reported that the maximum theoretical

TABLE 9.
AVAILABLE ELECTRON BALANCE AND YIELD RESULTS BASED ON INITIAL AND FINAL DATA

Study	η*	ξ*	$\eta+\xi$	η_{est}**	ξ_{est}**
IA	0.55	0.63	1.18	0.47	0.53
IB	0.55	1.03	1.58	0.35	0.65
II	1.22	1.19	2.41	0.51	0.49
III	0.70	0.42	1.12	0.63	0.37
IV	0.36	0.54	0.90	0.40	0.60
V	0.55	0.97	1.52	0.36	0.64
VI	0.42	0.68	1.10	0.38	0.62

* Results based on biomass, lactic acid, sucrose and stachyose measurements.

** Results based on biomass and lactic acid measurements.

value of η (η_{max}) is 0.305. Estimates of η_1, in Table 4, and estimates of η in Table 9 are greater than this maximum theoretical yield. Large yields also have been reported in other studies with lactic acid bacteria (Oner *et al.* 1984). Values of η can be estimated based on biomass and product measurements, viz., $\eta_{est} = \eta/(\eta + \xi)$. Values of η_{est} are also listed in Table 9. Removal of substrate measurements did not lower the estimates of η into the desired range ($\eta \leq 0.3$). Therefore, the direct microscopic count method of measuring growth contained error and was not precise. The complex nature of a soy-yogurt system makes experimental measurement difficult. Although studies have been reported in which yogurt bacteria were enumerated in bovine milk using fermentation vessels (Oner and Erickson 1986), no literature is available on enumeration of yogurt bacteria in actual set cup soy-yogurts. Consequently, refinement of biomass measurement techniques in soy-yogurt prepared by the set cup method is needed.

Another explanation for the large values calculated for η_1 is that during the initial stages of the yogurt fermentation, growth might have been aerobic rather than anaerobic. Oxygen was introduced into the soymilk as it was poured into the glass jars in which the fermentation was conducted. By transporting available electrons to oxygen in aerobic growth, the bacteria could realize a higher biomass yield.

Large variations of yield estimates from study to study indicated that sugar measurements may have contained error. Indeed, it was quite difficult to obtain precise and accurate HPLC data (see Part I, Buono *et al.* 1990, and Buono 1988).

Careful examination of the consistency of experimental measurements made on fermenting soymilks permits evaluation of the accuracy of the measurements and provides insight into possible alternate measurements that should be included in future studies.

CONCLUSIONS

Typical yogurt bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, have been shown to consume as much as 27% of the stachyose in soymilk after 7 h of fermentation as a mixed culture at 44°C. The bacteria required storage on soymilk for 336 h at -10°C to realize this utilization.

Lactobacillus bulgaricus and *Streptococcus thermophilus* grown as a mixed culture inoculum in a 1:1 ratio reached a pH of 4.5 in half the amount of time required for inocula prepared as two pure cultures. The fermentation time required to reach the desired pH was 3 h, which compares favorably to bovine yogurt fermentation times of 3–4 h.

Storage of lactic acid bacteria on soymilk for between 12 and 168 h at -10°C may have induced an enzyme or an enzyme system that permitted consumption

of stachyose by the bacteria. Storage on 11% nonfat dry milk for *Lactobacillus* and *Streptococcus* resulted in oligosaccharide formation when the bacteria were placed in soymilk.

Point estimates of the maximum specific growth rate of four soymilk mixed fermentations were made using the covariate adjustment technique. For bacteria subcultured on 11% NFDM, the μ_{max} estimates were 0.341 h^{-1} and 0.373 h^{-1}. For bacteria stored on soymilk for 168 h and subcultured on soymilk, μ_{max} was estimated at 0.296 h^{-1}. Finally, for bacteria stored and subcultured on 11% NFDM but prepared as a mixed culture inoculum, the μ_{max} estimate was 0.478 h^{-1}.

Available electron balances employed to evaluate data consistency indicated values greater than 1.00 for several soymilk fermentations. These balances suggest that alternate substrates other than carbohydrates may have been involved in growth and product formation.

ACKNOWLEDGMENT

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NOMENCLATURE

A_i	=	coefficients in Eqs. (17) through (19), i = 1, 2, 3.
CFU	=	colony forming unit.
CFUs	=	colony forming units.
C.I.	=	confidence interval.
Cov.	=	covariates.
P	=	lactic acid concentration at time = t, grams/liter
P_o	=	lactic acid concentration at time = t_o, grams/liter
Pt. Est	=	Point estimate.
S_{su}	=	sucrose concentration at time = t, grams/liter
S_{suo}	=	sucrose concentration at time = t_o, grams/liter
S_{st}	=	stachyose concentration at time = t, grams/liter
S_{sto}	=	stachyose concentration at time = t_o, grams/liter
t	=	time, hours
TA	=	titratable acidity: volume (mL) of 0.1 N NaOH added per mL soymilk sample, which was necessary to bring the pH to 7.0.
VCCL	=	viable cell count of *Lactobacillus*, CFU/mL
VCCS	=	viable cell count of *Streptococcus*, CFU/mL
X	=	biomass concentration at time = t, grams/liter

X_o = biomass concentration at time = t_o, grams/liter
Y = product biomass equivalents at time = t, grams/liter
Y_o = product biomass equivalents at time = t_o, grams/liter
Z = substrate biomass equivalents at time = t, grams/liter
Z_1 = covariate = $(\ln X - \ln X_o) + (\ln Y - \ln Y_o)$
Z_2 = covariate = $(\ln X - \ln X_o) - 2(\ln Z - \ln Z_o) + (\ln Y - \ln Y_o)$
Z_o = substrate biomass equivalents at time = t_o, grams/liter
γ = reductance degree, equivalents of available electrons per gram atom carbon
γ_b = $\gamma_{biomass} = 4.291$
γ_s = reductance degree of substrate = 4 equivalents of available electrons in substrate, either sucrose or stachyose, per gram atom carbon
γ_p = $\gamma_{lactic\ acid} = 4$
η = biomass available electron yield on substrate, equivalents of available electrons incorporated into biomass per equivalent of available electrons in the substrates
η_1 = biomass available electron yield on substrate during exponential growth, equivalents of available electrons incorporated into biomass per equivalent of available electrons in the substrates
μ = specific growth rate, h^{-1}
μ_{max} = maximum specific growth rate = μ during exponential growth, h^{-1}
ξ = product available electron yield on substrate, equivalents of available electrons incorporated into product per equivalent of available electrons in the substrates.
ξ_1 = product available electron yield on substrate during exponential growth, equivalents of available electrons incorporated into product per equivalent of available electrons in the substrates.
σ_b = weight fraction carbon in biomass, grams carbon/grams biomass
σ_p = weight fraction carbon in lactic acid product, grams carbon/grams lactic acid
σ_{su} = weight fraction carbon in sucrose, grams carbon/grams sucrose
σ_{st} = weight fraction carbon in stachyose, grams carbon/grams stachyose

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STORAGE STABILITY OF FRANKFURTERS CONTAINING CORN GERM PROTEIN[1]

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ABSTRACT

Storage stability of frankfurters containing 3% corn germ protein (CGP) defatted by supercritical CO_2 or hexane was determined by sensory characteristics and chemical and microbiological tests. Meaty aroma and meaty flavors scores of control and experimental samples decreased from 46.3–49.2 points after 3 days of storage to 34.2–42.3 points after 50 days of storage. Rates of increase in values of total volatile nitrogen (TVN), nonammonia amino nitrogen (NAAN), and 2-thiobarbituric acid (TBA) were similar in control and experimental frankfurters. These results of TVN, NAAN, and TBA tests indicated chemical changes in the protein and lipid components of frankfurters during storage. The incorporation of 3% CGP (both types) did not affect the rate of bacterial growth in frankfurters or stability during 50 days of storage at 3–4°C.

INTRODUCTION

Storage stability of meat products containing new food ingredients of plant origin is a critical factor for evaluating their value for practical usage. Plant protein incorporation in comminuted meat products (CMP) might have an effect on the proteolytic activity of microorganisms. Some bacteria have demonstrated specificity in their ability to utilize certain proteins. Combining plant and animal proteins may stimulate the growth of certain bacteria, which do not have the

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proteases necessary to grow on meat or plant protein medium alone (Sikes and Maxcy 1979).

Several reports of plant protein utilization have indicated that microbial growth became a significant factor for textured soy-ground beef (Bell and Shelef 1978), for soy protein flakes-ground beef (Harrison *et al.* 1983), and for sesame flour in fermented salami (Cruz and Hedrick 1985). Cottonseed protein has been found to inhibit growth of *Clostridium perfringens* and significantly increased generation times when added to meat proteins (Kokoczka and Stevenson 1976). Rhee *et al.* (1985) reported that no significant change of aerobic plate count occurred for ground beef with 3% glandless cottonseed flour after 3–6 days of displayed storage.

The soy proteins presently in use had been reported to be of good microbiological quality. Some soy proteins have such low bacterial counts that they may actually reduce the number of bacteria per gram of extended meat by a dilution effect (Stansbury 1975). Specifications from manufacturers usually permit less than log 4 bacteria/g of soy protein. There are controversial data related to the influence of soy proteins on storage stability of meat products. Seideman *et al.* (1977) tested ground beef alone and combined with texturized soy protein blends at 10, 20, and 30% levels. Plate counts of such formulations showed that the all-beef sample had the highest log count /g (6.0) after incubation for 10 days at 7°C. However, increased standard plate counts during storage of soy-ground beef formulation were associated with increasing levels of added soy proteins (Thompson *et al.* 1978; Keeton and Melton 1978).

Stansbury (1975) reported significant increases in aerobic and psychrophilic-mesophilic logarithmic counts in soy-extended ground beef with increasing levels of textured vegetable protein during storage at 0°C and 6°C. In one of the first articles on microbiological quality of extended meats, Judge *et al.* (1974) showed that the bacterial load was higher in blends made with soy flour compared to those containing only beef or soy protein concentrate. However, no significant difference in the bacterial counts was noted at the end of the storage period (7 days at 4°C).

Thermal processing during the production of CMP containing plant proteins may reduce the microbial count. However, the initial microbial loads and subsequent storage conditions can affect the shelf-life and quality of the product.

Compositional data and nutritional studies have shown that a high quality defatted corn germ protein (CGP) can be obtained by drying at low temperatures (Lucisano *et al.* 1984). Defatted CGP has been reported to have high water retention, fat binding, emulsifying capacity and emulsion stability (Lin and Zayas 1987b, c). CGP significantly increased water retention and yield of meat products (Zayas and Lin 1988). Because of low cost and high nutritional quality defatted CGP has considerable potential for use as a supplement in a variety of foods to provide new protein source (Lin and Zayas 1987a).

Studies on CGP utilization in sausage manufacturing have been reported (Zayas and Lin 1988). However, little work has been done on the storage stability of CMP containing CGP as a functional or substitute ingredient. This study was conducted to evaluate the stability of frankfurters stored 50 days as determined by sensory characteristics, chemical analysis: total volatile nitrogen, nonammonia amino nitrogen, and 2-thiobarbituric acid values; and microbial counts. Two types of CGP, hexane- or supercritical CO_2-extracted, were added to frankfurters.

MATERIALS AND METHODS

Sample Preparation

Corn germ protein (CGP) was defatted by hexane extraction. Supercritical CO_2 (SC-CO_2)- defatted CGP was obtained from USDA Northern Regional Research Center (Peoria, IL). The conventional oil extraction with hexane leaves certain lipids in the CGP and as the result of overheating reduces its nutritional quality (Phillips and Sternberg 1979). Christianson *et al.* (1984) used supercritical CO_2 method of defatting to extract oil from CGP and obtained a food grade quality defatted CGP. A flow chart of frankfurter production is shown in Fig. 1.

Fresh meat (50/50 beef trim [23.70%], boneless picnics [47.40%], and chicken meat [15.17%]) was ground through a 9.38 mm plate. To maintain a balance of protein and moisture contents, higher levels of water were added in experimental formulations. Water was added to the weight of meat in the formulations at levels of 23% for controls and 28 or 30% for samples receiving defatted CGP. Frankfurters were processed under commercial conditions at the Doskocils Co. plant (Pratt, KS). The formulation contained salt (2.84%), cure commercial frankfurter spice (Wiener seasoning, Griffith Labs, Alsip, IL), and 0.1% ascorbic acid.

After 3–4 min of chopping, SC-CO_2- or hexane-defatted CGP were added as powder at a level of 3% the weight of raw material with only 28% or both 28% and 30% added water, respectively. Defatted CGP was rehydrated simultaneously with the comminution of the sausage batter. Chopping was continued for a total of 10–12 min, until the batter temperature reached 14–15°C.

Batters were discharged from the chopper and stuffed into 24mm diameter casings, which were formed into links 11 cm in length and hung on a cooking rack. The frankfurters were cooked at 90°C for about 40 min to an internal temperature of 70°C in a commercial smokehouse. Then they were chilled by a 5 min cold shower and held in ice water for 20 min to reach 5°C before peeling. After peeling, the frankfurters were vacuum packaged and stored in a refrigerator at 3–4°C for up to 50 days. Three replications of each treatment were made.

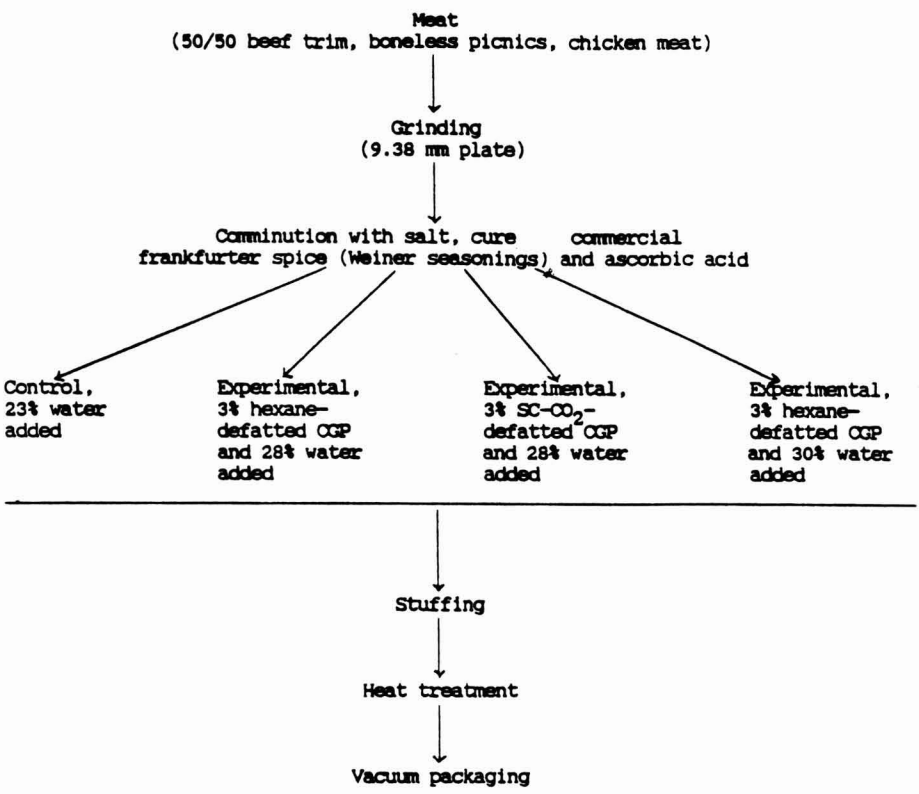

FIG. 1. FLOW CHART OF FRANKFURTER PRODUCTION

Sensory Evaluation

Frankfurters stored at 3–4°C for 3, 20, 40, and 50 days were used for sensory evaluation. Three links of frankfurters were selected randomly from each package and cooked 500 mL boiling, distilled, deionized water for 3 min. Then samples were sliced perpendicular to the long axis. Two 2 cm sections of each sample were presented to each panelist in a warmed custard cup covered with a watch glass. Panelists were asked to evaluate aroma first, then flavor.

A professional sensory panel was selected and trained according to the methodology developed by the Sensory Analysis Center at Kansas State University (KSU). Selected members of the panel after proper training, have been employed by the Department of Foods and Nutrition. A five-member panel was trained in four sessions to be familiar with the product descriptors of aroma and flavor. Reference samples containing none or 10% CGP were presented in four training sessions to represent the extremes on the scale. Beef bouillon, 2.2% salt solution, 0.5% liquid smoke, and corn flour were prepared for each panelist as reference samples throughout the study. Initial practice sessions focused on characteriz-

ing product aroma and flavor notes; later sessions, on quantifying these characteristics.

Scoring was done on a 60-point linear scale on a computer screen with descriptive anchors for each sensory parameter. Each scale was delineated at the midpoint. A computer program designed by the Department of Statistics, KSU facilitated random order of presentation. Four sets of samples were evaluated in each taste session: control and three experimental samples. Four replications were performed with two sessions for each storage interval.

Chemical Tests

Total volatile nitrogen (TVN) was measured by distillation using the method of Cob III *et al.* (1973). Amino nitrogen contents were determined by the trinitrobenzenesulfonic acid method of Camargo and Greene (1979) as modified by Kwan *et al.* (1983). A glycine standard dissolved in 5% tetrachloracetic solution was used. Nonammonia amino nitrogen (NAAN) was determined by subtracting the ammonia nitrogen from the total amino nitrogen. A 2-thiobarbituric acid (TBA) test was carried out following the method of Witte *et al.* (1970). TBA analysis was used as an index of fat oxidative rancidity. Chemical tests for TVN, NAAN, and TBA were performed at 1, 3, 6, 9, 20, 30, 40, and 50 days of storage.

Microbial Tests

A 10 gram sample of frankfurter was homogenized with 90 mL of saline solution in a blender. Five consecutive dilutions of the sample were made. The mesophilic test was performed on plate count agar (Difco Laboratories, Detroit, MI). Inoculated agar was incubated at 32°C for 48 h. The psychrotrophic test was carried out on plate count agar and then incubated at 7°C for 10 days. Viable cell counts were tabulated with replication of each sample.

Statistical Analyses

A completely randomized design was followed, and three batches per experimental treatment were randomly and independently processed and analyzed. One-way analysis of variance and least significant difference tests were conducted to test significance among treatment means (Steel and Torrie 1980)

RESULTS AND DISCUSSION

Sensory Evaluation

The mean scores for meaty aroma of frankfurters formulated with 3% corn germ protein (CGP) and control samples are presented in Table 1. There were

TABLE 1.
EFFECT OF STORAGE ON THE AROMA AND FLAVOR OF FRANKFURTERS CONTAINING CORN GERM PROTEIN (CGP)[c]

	Meaty aroma, time of storage, days				Meaty flavor, time of storage, days			
	3	20	40	50	3	20	40	50
Control[d]	49.2[a]	46.9[a]	42.3[b]	39.1[b]	49.6[a]	45.3[a]	39.4[b]	37.1[b]
SC-CO2 CGP[e]	46.9[a]	44.3[a]	38.6[b]	34.3[b]	47.3[a]	43.9[a]	38.2[b]	35.9[b]
Hexane CGP[f]	47.9[a]	45.8[a]	39.1[b]	37.4[b]	46.2[a]	42.3[a]	37.9[b]	34.7[b]
Hexane CGP[g]	46.3[a]	43.1[a]	36.7[b]	34.2[b]	46.4[a]	43.7[a]	38.6[b]	32.9[b]
MSE[h]	8.3	8.1	7.9	6.7	4.2	5.6	6.4	5.3

[a, b]Means with different letters in the same row are significantly different ($P < 0.05$)
[c]Frankfurters were evaluated using 6 in 60-point linear scale, 60 = strong, 0 = weak
[d]Control, without CGP; 23% water added
[e]Supercritical CO_2-extracted CGP, 3%; 28% water added
[f]Hexane extracted CGP, 3%; 28% water added
[g]Hexane extracted CGP, 3%; 30% water added
[h]Mean square error df = 12 N = 10

no differences in the intensity of meaty aroma after 20 days of storage. Meaty aroma scores decreased ($p < 0.05$) for all-meat control and three experimental samples after 40 days of storage. Control and experimental samples received scores of 46.3–49.2 points on a 60-point scale after 3 days of storage and 34.2–42.3 points after 50 days of storage. The effect of storage time on the meaty aroma was equal for control and experimental frankfurters with both types of CGP. Meaty aroma of all samples scored at the higher end of the scale (more than 30 points) should be recognized as acceptable.

The mean scores for meaty flavor of frankfurters formulated with 3% CGP and control samples, after 3, 20, 40, and 50 days of storage at 3–4°C, are presented in Table 1. Acceptable flavor was obtained in CGP-containing and control frankfurters by use of the same spice formulation. There were no significant differences in the intensity of meaty flavor ($P > 0.05$) after 20 days of storage. Meaty flavor scores of the three experimental and control frankfurters decreased after 40 days of storage. Experimental and control samples received scores of 46.2–49.6 points on a 60-point scale after three days of storage and 32.9–37.1 points after 50 days of storage. The general trend in flavor decrease was the same for control and experimental samples containing both types of CGP. All samples stored for 50 days had less meaty flavor (lower numerical scores) than samples stored for three days.

Total Volatile Nitrogen (TVN) and Nonammonia Amino Nitrogen (NAAN)

Storage stability of meat products was tested by the determination of biogenic amines, and total volatile and nonammonia amino nitrogen (Hui and Taylor 1983). These chemical indices of storage stability were used for vacuum packaged frankfurters stored at 3–4°C for 50 days. Total volatile nitrogen (TVN) and nonammonia amino nitrogen (NAAN) tests were carried out to confirm data from the sensory and microbiological analyses. Nonammonia amino nitrogen is an indirect indicator of the biogenic amine content in the product. Biogenic amine content is determined by the intensity of microbial and chemical changes of the samples during storage. Chemical changes include proteolysis of proteins with formation of polypeptides, dipeptides, amino acids, and end products of amino acids decomposition, particularly amines. Chemical tests such as the determination of biogenic amines, TVN, and ammonia nitrogen were used as spoilage indices in meat and fish products (Hui and Taylor 1983; Bryant *et al.* 1973).

Total volatile nitrogen of frankfurters was twice as much ($P < 0.05$) after 50 days of storage compared with the levels after three to six days of storage (Fig. 2). There was a permanent increase in TVN during the storage period, which was similar in the all-meat control and three experimental frankfurters. There was no significant difference ($P > 0.05$) in the rate of increase and levels of TVN between control and experimental samples, between samples containing

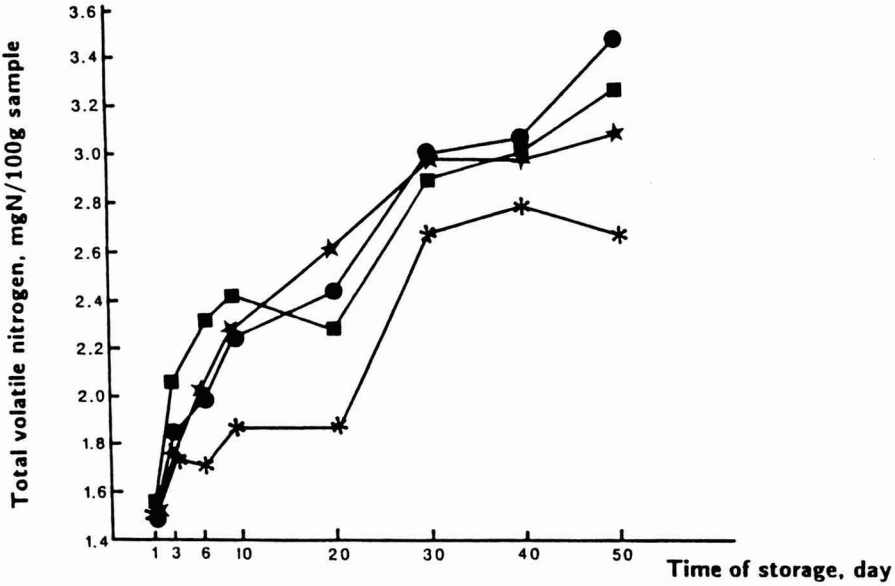

FIG. 2. EFFECT OF STORAGE ON THE TOTAL VOLATILE NITROGEN CONTENT OF FRANKFURTERS

● Control, 23% water added
■ 3% SC-CO_2 CGP, 28% water added
★ 3% hexane CGP, 28% water added
✱ 3% hexane CGP, 30% water added

hexane-defatted CGP or supercritical-CO_2 defatted CGP, or between samples containing different amounts (28 and 30%) of added water.

As a result of the acceleration of chemical changes, there was a significant increase ($P < 0.05$) in NAAN of frankfurters between 6 and 10 days of storage (Fig. 3). No significant ($P > 0.05$) differences were found in the levels of NAAN between 10 and 40 days of storage. However, there was an increase ($P < 0.05$) in the levels of NAAN after 50 days of storage, compared with the levels after three to six days of storage. The rate of NAAN increase was the same ($P < 0.05$) for the all-meat control and experimental samples of frankfurters, containing hexane- or supercritical-CO_2-extracted CGP (Fig. 3). The amount of added water (28 and 30%) in formulations of frankfurters containing hexane-defatted CGP did not affect the level of NAAN or the rate of NAAN increase during storage. Results of these tests of the TVN and NAAN contents indicated chemical changes during storage of frankfurters.

Several researchers have reported that some plant proteins, for example, soy protein, retarded rancidity development in meat products (Kotula *et al.* 1976). There were no significant differences ($P > 0.05$) in TBA values of frankfurters

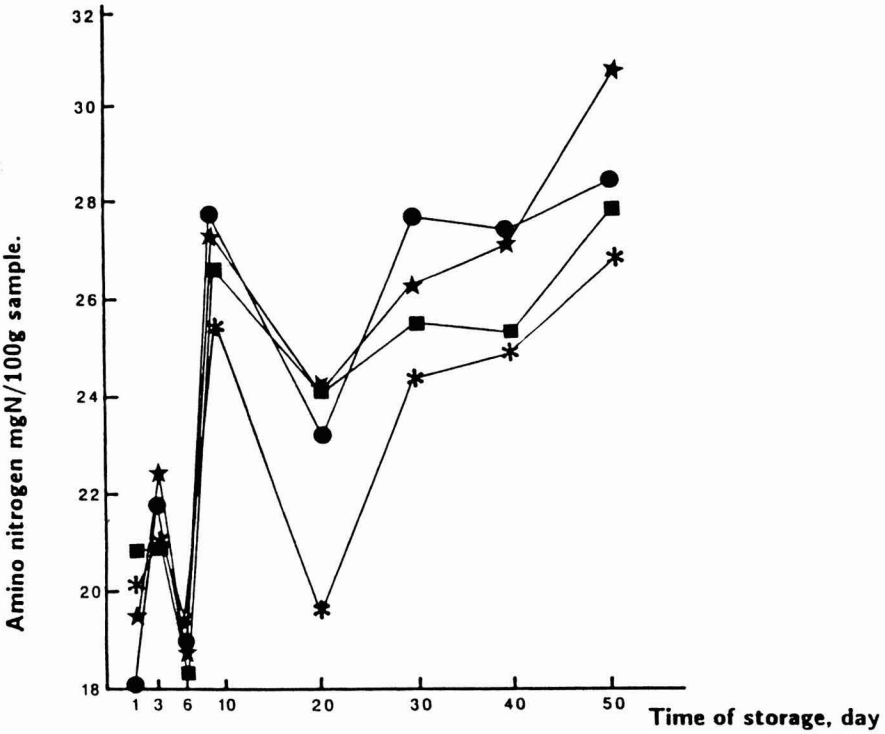

FIG. 3. EFFECT OF STORAGE ON NONAMMONIA AMINO NITROGEN CONTENT OF FRANKFURTERS

● Control, 23% water added
■ 3% SC-CO_2 CGP, 28% water added
★ 3% hexane CGP, 28% water added
* 3% hexane CGP, 30% water added

after processing, at the beginning of storage, and after 50 days of storage (Fig. 4). The general trend of changes in TBA values was the same for the all-meat control and experimental samples. The drawback of the TBA method is the possibility of interfering reactions, which can alter the pink color formation because malonaldehyde is reactive with other food components (primary amino groups of proteins). A decrease of TBA values with increasing storage has been reported (Witte *et al.* 1970). The net result is that TBA values may appear to decrease over time, although lipid oxidation is known to continue.

Microbiological Quality

A comminuted meat products (CMP) provide an excellent medium for microbial growth and become contaminated as a result of grinding an mixing during

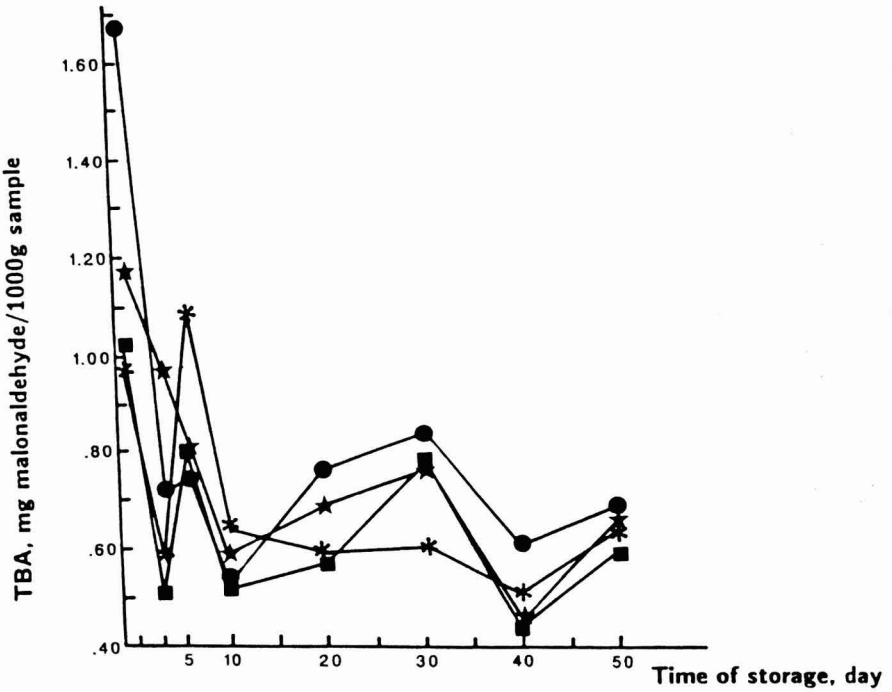

FIG. 4. EFFECT OF STORAGE ON THE TBA VALUES OF FRANKFURTERS

processing. The overall microbial quality of CMP depends upon the microbial counts of the raw material, seasonings, and additives; sanitation during processing, especially grinding, comminution, and stuffing; type of packaging; time and conditions of storage.

Mean values of logarithmic bacterial numbers (mesophilic and psychrotrophic) per 1g for three CGP-containing and one control samples during storage are presented in Fig. 5 and 6. Total plate counts increased with length of the storage period, but a significant increase ($P < 0.05$) of total plate counts (mesophilic and psychrotrophic) was found only after 40 days of storage. Bacterial counts of all samples ranged from 1.3×10^3–1.4×10^3 bacteria/g at day 0 to 6.1×10^6–1.6×10^7/g after 50 days of storage. After 50 days of storage, there were generally no significant differences ($P > 0.05$) between formulation treatments for microbial counts (mesophilic and psychrotrophic) (Fig. 5 and 6). However, mesophilic counts for frankfurters containing 3% hexane-defatted CGP and 30%

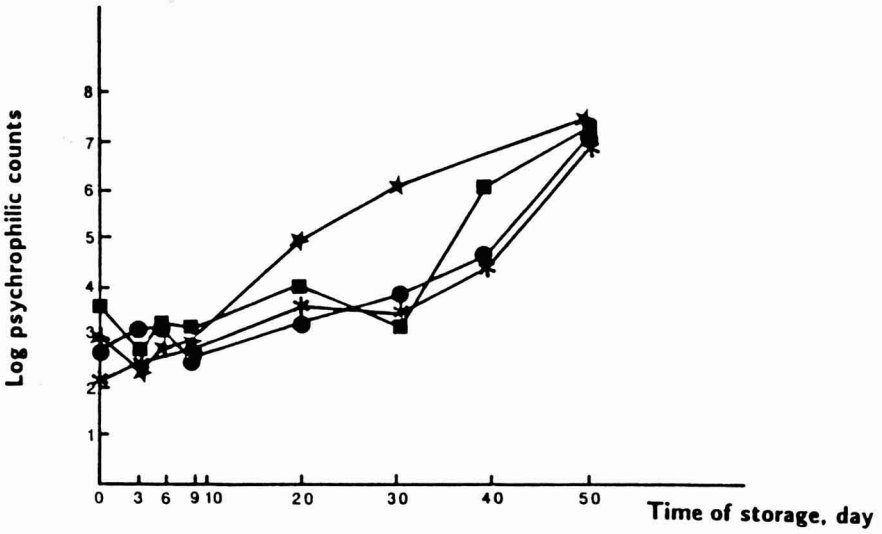

FIG. 5. LOGARITHM OF VIABLE CELL COUNT OF PSYCHROTROPHIC COUNTS DURING STORAGE OF FRANKFURTERS

● Control, 23% water added
■ 3% SC-CO_2 CGP, 28% water added
★ 3% hexane CGP, 28% water added
✱ 3% hexane CGP, 30% water added

added water were lower ($P < 0.05$) than other experimental and control samples (Fig. 6). As illustrated in Fig. 5 and 6, there were similarities between experimental and control samples. Log numbers of mesophilic and psychrotrophic bacteria per gram of three experimental and control frankfurters increased with storage time from 10 to 50 days (Fig. 5 and 6). In all four treatments, the rate of increase accelerated after 10 days of storage. The rate of increase and a total log counts of psychrotrophic and mesophilic bacteria were the same for control samples with 23% water added during formulation and experimental samples with 3% of two different CGP (hexane-and SC-CO_2-defatted) and 28% water added during formulation. However, the rate of increase was higher with hexane-defatted CGP added at 3% level and 28% water. These results indicated that the addition of 2% more water in the formulation did not affect the stability of the system.

Logs of mesophilic and psychrotrophic bacterial counts per gram were higher ($P < 0.05$) after 50 days of storage that after 10 days. The relationship between log of mesophilic and psychrotrophic bacteria counts and storage time was significant ($P < 0.05$). Psychrotrophic counts were found not to be significantly higher than mesophilic counts after 50 days of storage (Fig. 5 and 6). They were

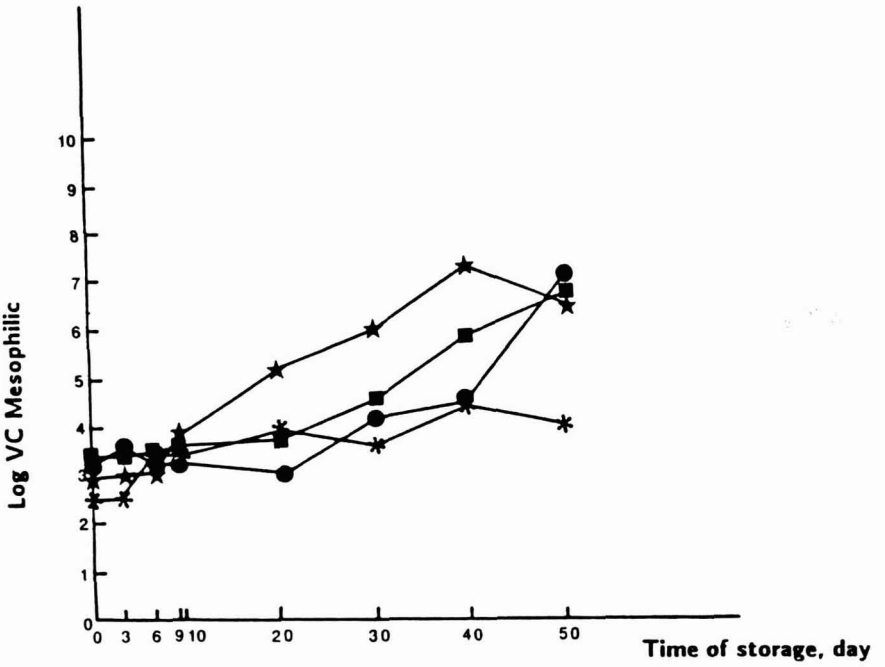

FIG. 6. LOGARITHM OF VIABLE CELL COUNT OF MESOPHILIC ORGANISMS DURING STORAGE OF FRANKFURTERS

in the range of log 6–log 7. After storage at 3–4°C for 50 days, psychrotrophic counts were approximately 5.0 log/g higher than after processing; mesophilic counts were 3.7 log/g higher. A mesophilic count is recommended in addition to the psychrotrophic count as an indication of initial meat quality and potential sanitation problems.

Storage tests clearly showed no significant differences ($P > 0.05$) in shelf stability between frankfurters containing CGP and the all-meat control stored at 3–4°C for 50 days. There were no specific characteristics of spoilage at the end of the storage period. The acceptable limit for storage before control and experimental frankfurters were permitted for consumption was 40 days. After 30–40 days, experimental and all-meat control frankfurters stored at 3–4°C were microbiologically stable.

CONCLUSIONS

The incorporation of 3% corn germ protein (CGP) defatted by hexane or supercritical-CO_2 extraction (SC-CO_2)- methods did not affect storage stability of frankfurters, as determined by sensory characteristics and chemical and bacteriological tests. There were no differences in the intensity of meaty aroma and meaty flavor of frankfurters after 20 days of storage. Meaty aroma and meaty flavor scores of all samples decreased after 40 days of storage. The general trend for decrease of meaty aroma and meaty flavor was the same for control and experimental frankfurters. Scores for meaty aroma and flavor of all samples at the higher end of the scale (more than 30 points) should be recognized as acceptable.

Total volatile nitrogen (TVN) increased after 50 days of storage. As a result of the acceleration of chemical changes, there was a significant increase in NAAN of frankfurters between 6 and 10 days of storage but no significant difference was found between 10 and 40 days. The general trends for rates and levels of increases in TVN and NAAN were similar in the all-meat control and three experimental frankfurters containing SC-CO_2- or hexane-extracted CGP and different amounts of added water.

There were no significant formulation treatment effects on TBA values. No significant differences were found in TBA values of frankfurters after processing and at the beginning and after 50 days of storage. The trend for changes in TBA values was the same for control and experimental frankfurters. Results of TVN, NAAN, and TBA tests indicated chemical changes in protein and lipid components of frankfurters during storage.

The incorporation of 3% CGP defatted by hexane or supercritical-CO_2 extraction methods did not affect the rate of bacterial growth in frankfurters during 50 days of storage at 3–4°C. The lack of difference in bacterial growth curves between control and experimental samples indicated that there was no additional microbial contamination during CGP incorporation in frankfurters formulations. Microbial counts, both mesophilic and psychrotrophic, of the all-meat control and experimental samples increased between 10 and 50 days of storage.

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THERMAL DEATH KINETICS OF *B. STEAROTHERMOPHILUS* IN THIN FILM SCRAPED SURFACE HEAT EXCHANGER

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ABSTRACT

A cascade thin film scraped surface heat exchanger, having sterilizer, regenerator and cooler sections was tested for thermal death kinetics of B. stearothermophilus *spores in milk. A sterilizing effect of 6.15 was obtained at 152°C for a holding time of 1.25 s. D values of 1.18, 0.75, 0.25 and 0.20 s were obtained for* B. stearothermophilus *at 143, 145, 150 and 152°C, respectively. The Z-value obtained was 10.1°C.*

INTRODUCTION

Thermal processing is one of the most important methods for preserving food materials. By raising processing temperatures and reducing the time, the bactericidal effect of the process is increased while at the same time the undesirable chemical changes which cause browning, brought about by high temperature are reduced. This is the basis for high temperature/short time and ultra high temperature (UHT) processing of milk.

For evaluating UHT sterilization process, heat resistant spores only need consideration. All vegetative organisms and relatively less heat resistant spores are destroyed by UHT process. Spores of *B. subtilis* or *B. stearothermophilus* are commonly used in experimental work as they are most resistant mesophilic and thermophilic spores respectively and likely to survive in this method of processing. Because of small number of heat resistant spores generally present in raw milk, the number of survivors from an effective sterilizing process is so small that it is unlikely to be detected by conventional bacteriological analysis. Galesloot (1956) suggested that effectiveness of UHT processes, should be tested by using raw milk inoculated with a large number of heat resistant spores detectable by conventional counting method. He proposed that effectiveness

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of process can be measured by sterilizing effect. A comprehensive report was published for direct heating UHT process. (In Sweden, Lindgren and Swartling 1963 and Thome *et al.* 1964, in Denmark, Moller-Madsen *et al.* 1965a). A similar report was also published on the Paasch and Silkberg direct UHT plant (Moller-Madsen *et al.* 1965b). Most recent and comprehensive data on thermophilic spores in milk can be found in Davies *et al.* (1977); Perkin *et al.* (1977) and Horak and Kessler (1981). Apart from it a study on reaction kinetics is available in Burton *et al.* (1959); Miller and Kandler (1967); Shehata and Collins (1972); Konietzko and Reuter (1980) and Anap (1985).

One method suited for obtaining the ultra high temperatures and short times required to produce a satisfactory product is by processing milk in a scraped surface heat exchanger (SSHE). Because of its unique flow characteristics, fouling and burn on problems are minimized. In addition turbulence generated by agitation and scraping in the bulk of fluid near the heat transfer surface makes heat transfer more efficient.

This paper describes the reaction kinetics of *B. stearothermophilus* spores in thin film SSHE.

MATERIALS AND METHODS

(1) Milk

Fresh buffalo milk was collected from National Dairy Research Institute (NDRI), Farm.

(2) Test Organism

Spores of strain *B. stearothermophilus* (C-953) obtained from National Collection Centre, Dairy Bacteriology Division, NDRI.

For cultivating the above organism, a media containing various nutrients was prepared. The composition of media was as follows:

Tryptone	3 g
Beef Extract	1.5 g
Agar-Agar	25 g
Peptone bacteriological	6 g
Yeast Extract	3 g
Solution containing .001% w/v Mn^{++}	1 mL
Distilled Water	1 L
pH	7.0 ± 0.1

(3) Preparation of Nutrient Agar Media

Nutrient agar media was prepared by mixing all its ingredients in distilled water. It was heated in water bath at 100°C and pH was adjusted to 7.0 ± 0.1. The media was autoclaved at 121°C for 20 min for sterilization and stored for subsequent use.

(4) Preparation of Test Organism

B. stearothermophilus strain was inoculated on nutrient agar slant. Actively growing vegetative cells were prepared on large scale by inoculating the spore strain on the surface of nutrient agar. Cells from slant were used to inoculate a flask containing 50 mL of nutrient broth. After 24 h of incubation at 55°C, cells were removed from the incubator. A portion around 100 mL nutrient agar was poured in several 500 mL conical flasks and Raux bottles. After the medium was autoclaved and was allowed subsequently to solidify, agar in each bottle was then inoculated by spreading 0.5 mL of cell suspension evenly over the surface. The spore crop was grown on the surface of agar nutrient for 3 days at 55°C. Sterile distilled water (10 mL approx) was added to each bottle simultaneously scraping gently the agar surface with sterile rod. The liquid containing cells from all bottles was pooled, centrifuged and washed thrice with distilled water. The spores obtained from centrifugation were suspended in water and stored at 4°C approximately for 2 months.

Determination of Spore Count

The number of spores in raw buffalo milk, inoculated milk and spores suspension liquid were determined by colony count method. The composition of media used for counting *B. stearothermophilus* spores (BS media) is described as follows:

Tryptone	3 g
Peptone	6 g
Yeast Extract	3 g
Beef Extract	1.5 g
Agar-Agar	22 g
Bromocresol purple	0.04 g
Starch	2.0 g
Distilled water	1 L
pH	7.0 ± 0.1

Preparation of Media

The BS media was prepared by mixing all its ingredients in distilled water. It was heated in water bath at 100°C and pH was adjusted to 7.0 ± 0.1. It was autoclaved at 121°C for 20 min for sterilization and stored for subsequent use.

Experimental Set-up

The schematic diagram of experimental set-up is shown in Fig. 1. The experimental set-up consisted of a feed tank, feed pump, sterilizer, regenerator, cooler, driving motor, liquid sealing tube and aseptic sampling unit. The experimental set-up was sanitized and sterilized according to procedures described elsewhere (Dodeja 1987).

Milk was first pumped to regenerator section. The temperature of raw milk leaving the regenerator was in the range of 65° to 70°C. It was raised to temperatures in the range of 143° to 152°C in the sterilizer. Holding times of 0.75, 1.0 and 1.25 s were obtained from mass flow rates of 1.33×10^{-2}, 1.67×10^{-2} and 2.23×10^{-2} kg/s, respectively. Equipment was manipulated to obtain different time-temperature combinations in the range mentioned.

Experimental Procedure

The total number of spores present in the raw milk and spore suspension were determined. Twenty liters of raw buffalo milk were inoculated with predetermined quantity of *B. stearothermophilus* suspension to raise the spore level in milk to 10^8/mL. Milk was filled in feed tank. The pumping unit was switched-on to admit milk into the sterilizer. Immediately Nitrogen and steam were allowed to enter into sterilizer and its jacket, respectively. Motor of rotors was switched on. When milk was noticed at the outlet of the cooler, valves (V_5) and (V_{10}) were regulated in such a manner that liquid seal was formed at the outlet of sterilizer. The mass flow rate of milk was adjusted by regulating valves (V_2) and (V_3). The pressure of nitrogen in the sterilizer was also regulated to prevent boiling of milk. The chilled water was allowed to enter the jacket of cooler. When steady state conditions were attained, all the process variables were recorded. Milk was collected in aseptic sampling bottles. At the end of operation, the experimental set-up was rinsed with water and then cleaned in accordance with standard procedure.

Similar trials were conducted for processing temperatures of 143°, 145°, 150°, and 152°C for holding times of 0.75, 1.0 and 1.25 s.

Spore survivors in all samples collected for above conditions were estimated.

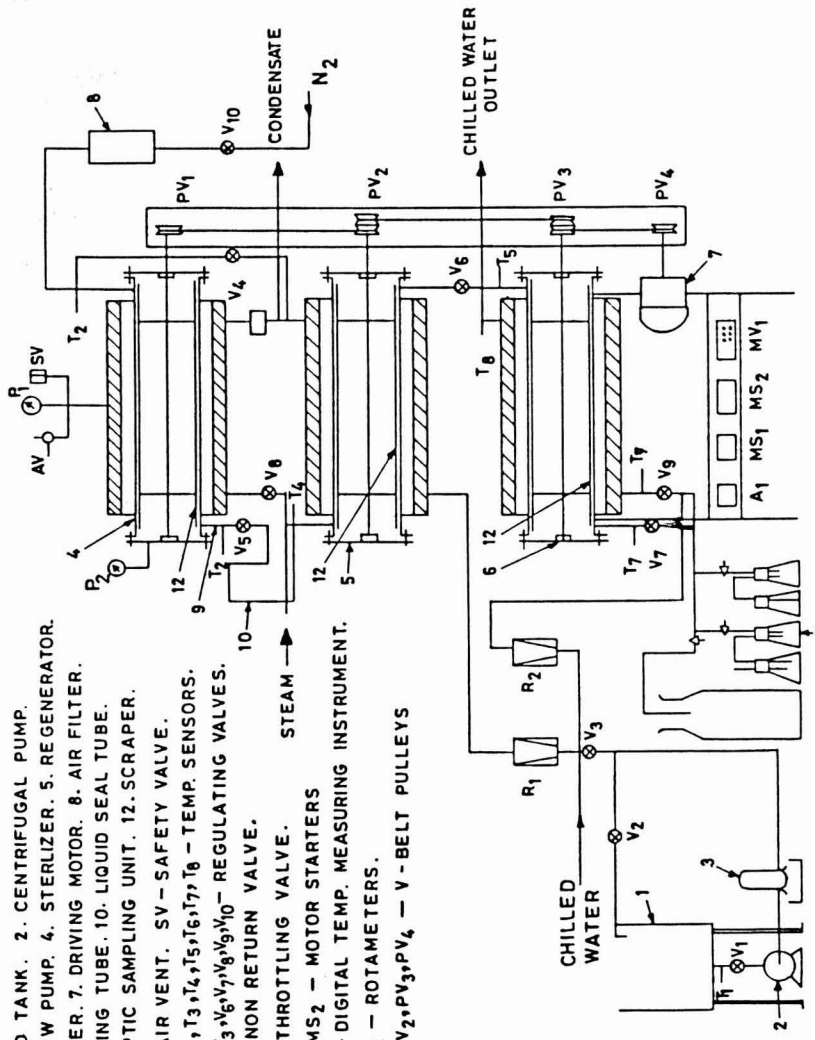

FIG. 1. SCHEMATIC DIAGRAM OF CASCADE THIN FILM SSHE

RESULTS AND DISCUSSION

The sterilizing effect $\log_{10}$ (Initial spores/survival spores) obtained for UHT processing at different time-temperature combinations is indicated in Table 1. The highest sterilizing effect of 6.15 is noticed at 152°C and holding time of 1.25 s. In commercial practice, a wide range of sterilizing effects varying between 2 to 8 are commonly employed depending on the number of initial spores present in milk and required sterility to be achieved. Ridgway (1955, 1958) found a seasonal variation in number of thermophilic resistant spores with a minimum in summer and maximum in winter. Franklin *et al.* (1956) too confirmed the seasonal variation in spores. In a detailed study carried out by Solanki (1987) which took into account different seasons, climate, locations and specific situations, the maximum value of total spore count was observed as 100/mL. Survivals of 1 spore/100 L (1% acceptance level) has been recommended (personal communication, National Dairy Development Board, India). This places requirement of maximum sterilizing effect as 7. The present investigation reveals that sterilizing effect up to 6.15 could be obtained by employing a thin film SSHE.

The thermal death time (TDT) or D value, can easily be determined from the logarithmic plot of the number of organisms against time. Figure 2 is a plot for logarithm of number of survivors against holding time at different processing temperatures. The D value can directly be read from the plot. The D values so

TABLE 1.
STERILIZING EFFECT OF EXPERIMENTAL SET-UP
FOR *B. STEAROTHERMOPHILUS* SPORES

Temperature	Holding time (seconds)	Colony count cfu/ml	Sterilizing effect
143	0.75	22.98×10^6	0.63
	1.00	13.97×10^6	0.85
	1.25	83.94×10^5	1.07
145	0.75	98.27×10^5	1.00
	1.00	47.28×10^5	1.32
	1.25	20.73×10^5	1.68
150	0.75	95.78×10^3	3.01
	1.00	94.64×10^2	4.02
	1.25	111.49×10^1	4.95
152	0.75	17.00×10^3	3.77
	1.00	85.00×10^1	5.07
	1.25	70.00×10^1	6.15

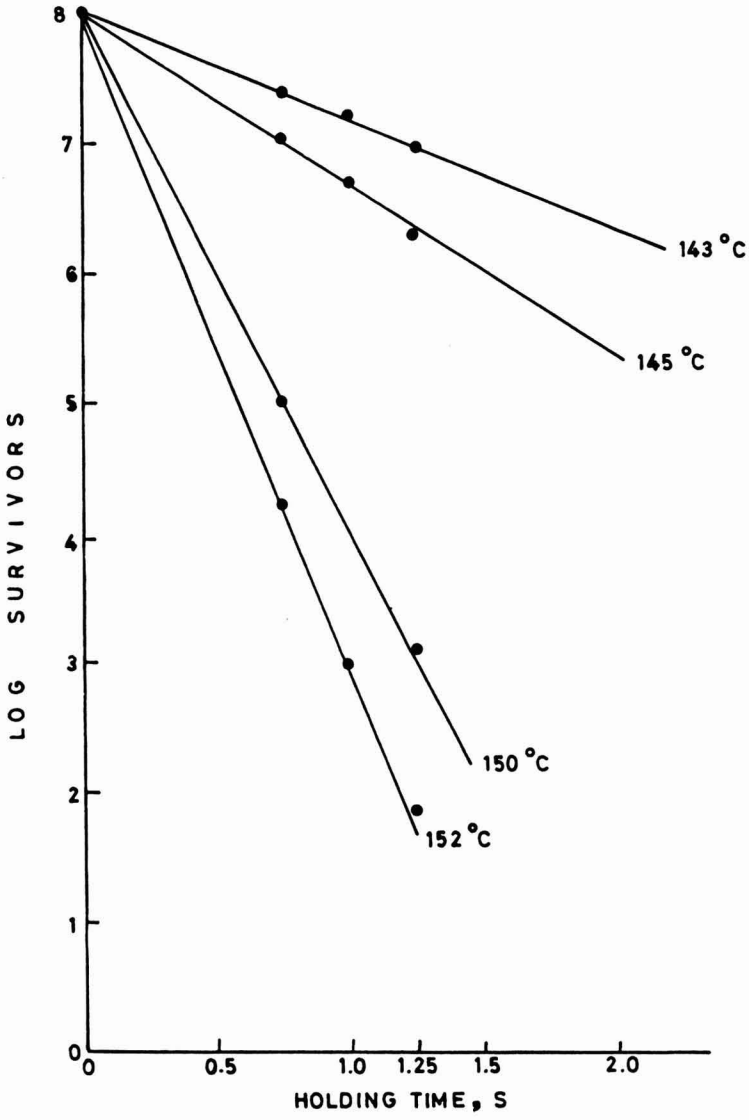

FIG. 2. THERMAL DEATH PLOTS FOR *B. STEAROTHERMOPHILUS* AT DIFFERENT TEMPERATURES

determined at various temperatures are shown in Table 2. Figure 3 is a plot for log D against temperature. From this figure the value of Z for *B. stearothermophilus* is obtained as 10.1°C.

It was observed that D values of present study are lower than those obtained by Davies *et al.* (1977). For a temperature range of 143 to 152°C, they reported

TABLE 2.
D VALUES FOR DESTRUCTION OF *B. STEAROTHERMOPHILUS* SPORES

Process Temperature °C	D-value (seconds)
143	1.18
145	0.75
150	0.25
152	0.20

D values between 1.73 to 0.32 s. The published literature indicates various factors which effect the heat resistance of the spores. This subject was systematically reviewed by Brown and Ayres (1982). These factors are firstly those affecting sporulation, secondly those arising due to the condition of storage of spore suspensions, thirdly those affecting the conditions during the heat treatment and lastly those effecting recovery of survivors i.e., type of recovery media employed together, with incubation temperature (Prentice and Clegg 1974).

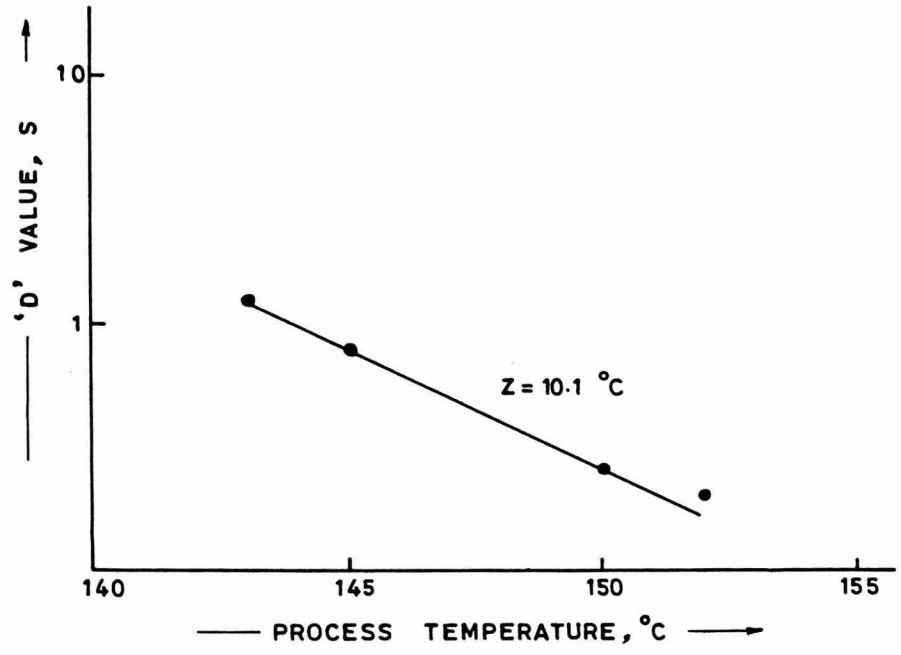

FIG. 3. DECIMAL REDUCTION TIME PLOT FOR *B. STEAROTHERMOPHILUS* SPORES DURING UHT PROCESSING OF MILK

Davies *et al.* (1977) used TH24 (NCDO 1096) strain of stearothermophilus with sporulation temperature 60°C. In present study a sporulation temperature of 55°C was used instead of 60°C used by Davies *et al.* with different thermophilus strain C-953. The sporulation temperature has considerable effect on D-values. It becomes double when the sporulation temperature is increased from 50° to 60°C (Cook and Gilbert 1968). The type of strain also showed variation in D-value (Brown and Ayres 1982). Further the work reported by Davies *et al.* was obviously done with spore suspension of *B. stearothermophilus* in cow milk while present investigation was purposely done employing Buffalo milk. In view of above mentioned reasons variation in D values could be expected. It may be seen that value of Z obtained during present investigation was within the range of values reported in literature.

The D and Z value facilitate determination of time-temperature combinations necessary for obtaining the required sterility in the final product. The thermal death kinetics parameters determined by this study should prove useful in selection of process conditions for UHT sterilization of milk in cascade thin film SSHE.

CONCLUSIONS

A study on Reaction Kinetic of *B. stearothermophilus* in cascade thin film scraped surface heat exchanger was carried out. At 152°C and for holding time of 1.25 s, a sterilizing effect of 6.15 was obtained. D values of 1.18, 0.75, 0.25 and 0.2 s were obtained at 143°, 145°, 150° and 152°C, respectively. Even at high processing temperatures, such as 152°C, the quality of product was found satisfactory. Hardly any fouling on heating surface was observed at these temperatures. These D and Z values reported should be helpful in selection of process parameters for thermal destruction of *B. stearothermophilus* spores in cascade thin film scraped surface heat exchanger.

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CHARACTERISTIC TEMPERATURES DETERMINATION FOR STRAWBERRY FREEZING AND THAWING

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ABSTRACT

Differential scanning calorimetry (DSC) was employed to determine the freezing and thawing behavior of two strawberry cultivar, Tioga and Florida, since reported thermophysic values for them were not found. Supercooling, complete solidification, minimum incipient melting, maximum incipient melting, and onset temperatures with a reliable range of accuracy were evaluated from DSC curve. The cryoscopic temperature, the most difficult determination from the thermograms, was calculated following a method that used only experimental DSC data. This temperature was dependent on the unbound water and soluble solids concentration, therefore the value obtained for each cultivar was different: $-0.78°C$ for Florida cultivar with 89% of water and $-1.41°C$ for Tioga cultivar with 87% of water. Temperatures were compared with literature values for other strawberry cultivars to analyze the validity of the results.

INTRODUCTION

Several food preservation technologies are carried out by decreasing temperature. Knowledge of chemical and physical changes with this variable during the freezing process allows one to set up the conditions required to ensure the quality of the product and the efficiency of the equipment.

Temperature versus time or temperature versus freezable water relationships are widely used for following solid food freezing or thawing. They can provide temperature limits points or bases for recommending cooling rates. Accurate temperature measurements indicating changes in solid food are also useful in obtaining models that simulate design conditions.

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Many experimental investigations have been made to determine the calorimetric behavior of foods. The most widely known are enthalpy-temperature determinations (Heldman 1974), phase equilibrium studies in acqueous solutions (Guegov 1960), different thermal analysis applications to measure the unfreezable water (Duckworth 1971; Simatos *et al.* 1975; Ross 1978), specific heat calculations (Roos 1986) and also thermal methodologies for studying heat treatments in foods (Parsons *et al.* 1986). Several models of thermal properties have been tested correlating calorimetric data (Heldman 1974; Chang *et al.* 1981; Schwartzberg 1977) and very useful data recopilations were made (Guegov 1981; Dickerson 1968).

Temperature measurements can be easily assessed by Thermal Analysis (TA) using simple thermocouples. Differential Thermal Analysis (DTA), a more elaborated technique, has been applied to improve accuracy but it has not been frequently used in food technology. However, Differential Scanning Calorimetry (DSC), which measures the absorbed or released heat change, has been lately carried out in food laboratories.

DSC has been used for evaluating thermal properties in foods. Heat capacity values and enthalpy changes have been directly obtained with DSC equipment; characteristic temperatures can be assessed, but with additional precautions. Simatos *et al.* (1975) attributed the problem in detecting temperatures when phase changes occur to the small size of the sample and suggested DTA experiment as a more accurate method. Nevertheless, Guegov (1981) asserted that certain processes accompanied by a substantial endothermic effect, such as intensive melting, can be accurately registered by DSC.

The absence of thawing superheating phenomenon in foods and the nucleation of the liquid phase from solidified phases without delay are other reasons for thinking that DSC heating temperature determinations are reliable.

The object of this work was to obtain strawberry freezing and thawing temperatures using only DSC measurements; and to analyze the results according to the composition of the cultivars and basic principle given in the literature. The initial freezing temperature was calculated proposing a method that uses only calorimetric DSC data.

MATERIALS AND METHODS

Two commercial strawberry cultivars were tested, Florida and Tioga. Soluble solids and water contents were determined following AOAC methods (AOAC 1984).

0.5 kg of strawberries (Tioga cultivar) were loaded into a stainless steel box at ambient temperature, and placed in a constant temperature freezer chamber at $-29°C$ for TA experiments. A Chromelalumel thermocouple and a Yokogawa

Hokushin Electric Recorder YEW-ER 187 were used to measure and register temperature evolution. The estimated initial freezing rate was about 0.08 K/min.

A Metler TA 3000, DSC 30 was used for DSC measurements. Twenty mg product samples with and without seeds were placed in standard aluminum pans with a pierced lid. An empty aluminum pan was used as reference sample; liquid nitrogen was used for cooling. Cooling or heating rates of 2 K/min were used. Temperature and heat calibrations were made following the equipment manufacturers' suggestions.

The temperature range was from -50°C to 20°C for both heating and cooling measurements. The lowest temperature was selected after having found in several runs that detectable heating peaks did not go below -50°C. The only temperature event provided by the equipment was the thawing peak temperature, T_{ph}, in heating runs. Others thermal characteristics were evaluated from thermograms.

At least three experimental runs were performed to obtain different temperature records and average values. Maximum and minimum temperatures were taken to define deviation values.

Technical nomenclature was defined following the current literature.

T_{sc} read from the DSC freezing curve, was the temperature at which latent heat evolution began. T_{im} and T'_{im} were taken from heating runs where the curve clearly had left the initial base-line, indicating that some latent heat was evolved. The "onset-of-melting" temperature, T_m, calculated by the method proposed by Ross (1986), was the intersection of the base-line and the large endothermic peak line prolonged.

T_i was obtained from DSC measurement results by the alternative method derived from the following effective heat capacity equation for foods in a freezing process proposed by Schwartzberg (1981)

$$C_e = C_f + \frac{n_w \, DH_o \, (T_o - T_i)}{(T_o - T)^2} \tag{1}$$

This equation can be expressed:

$$C_e = a' + \frac{b'}{(T_o - T)^2} \tag{2}$$

Where a′, b′ are parameters for a given product.
From (1) and (2):

$$T_i = T_o - \frac{b'}{n_w \, DH_o} \tag{3}$$

T_i can be calculated by providing b′ and the frozen water n_w.

Pairs of (C_e, T) values measured with the Differential Scanning Calorimeter were correlated with a regression optimization method to calculate coefficients a′ and b′.

An appropriate enthalpy change DH, for all water frozen was determined from the DSC experience and used in the following equation as Roos (1986) to determine n_w.

$$n_w = \frac{DH_f}{DH_w} \tag{4}$$

where DH_f and DH_w were sample and water latent heat between -40°C and 10°C, respectively. Neither initial water content nor solute concentration in food were needed to determine T_i.

T_i were also obtained using others methods. Guegov (1981) derived a general relationship for T_i in centigrade expressed by:

$$T_i = 0.36 - 0.175\,E \tag{5}$$

and Chang *et al.* (1981) recommended to determine T_i in vegetables and fruits by:

$$T_i = 14.41 - 49.19\,n_{wo} + 37.07\,n_{wo}^2 \tag{6}$$

where E is the soluble solid weight percent and n_{wo} is the total water content (weight fraction).

The chart of T_i vs E proposed by Guegov (1981) for determining T_i of different products was also used.

RESULTS AND DISCUSSION

The soluble solids contents were about 5% w/w for both cultivars and water contents were about 89% w/w and 87% w/w for Florida and Tioga, respectively.

The characteristic TA Temperature-Time curve for strawberry freezing is shown in Fig. 1. A constant initial freezing temperature of -1.8°C with an estimated error of ± 0.5°C, was read from thermograms.

Representative DSC curves during strawberry freezing and thawing respectively, are shown in Fig. 2 and 3. T_{cs}, T_{im}, T'_{im}, T_{sc} and T_m are indicated.

Values of T_i and T_{sc} are listed in Table 1. The values of b′ were about 231 and 379 J C/g, and n_w were 0.839 and 0.802 w/w for Florida and Tioga strawberries, respectively (Delgado *et al.* 1988). Values of T_{im}, T'_{im}, T_m and T_{ph} are

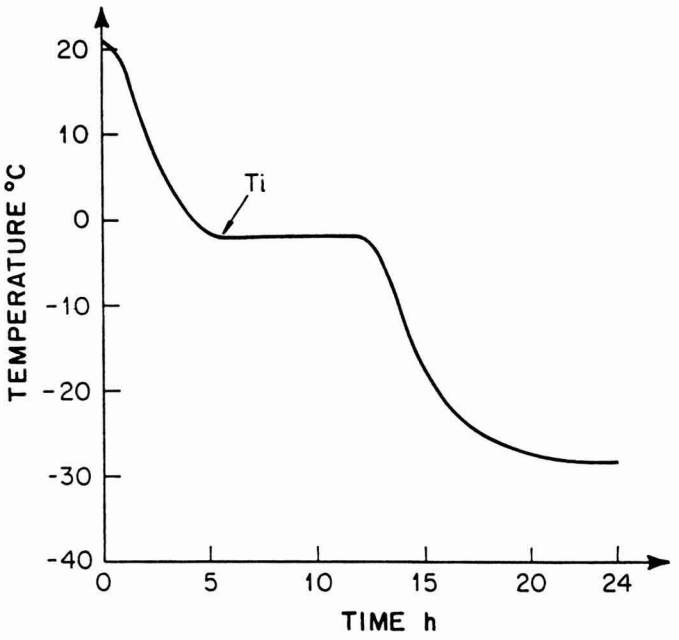

FIG. 1. TYPICAL TEMPERATURE-TIME CURVE FOR THE STRAWBERRY FREEZING

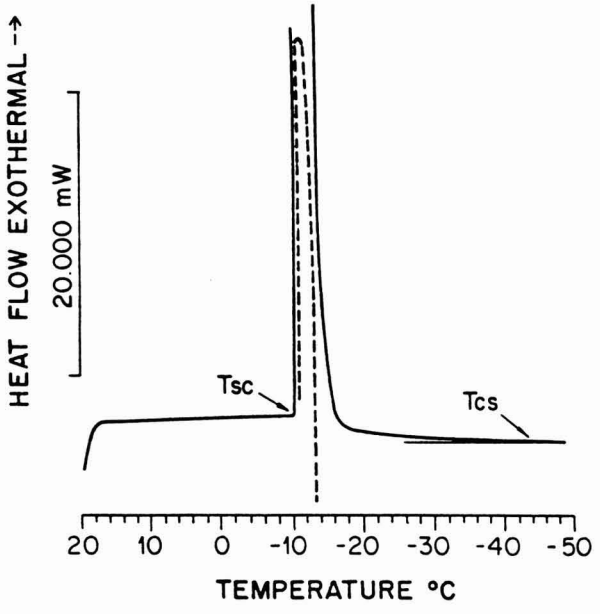

FIG. 2. DSC COOLING THERMOGRAM FOR FRESH STRAWBERRY

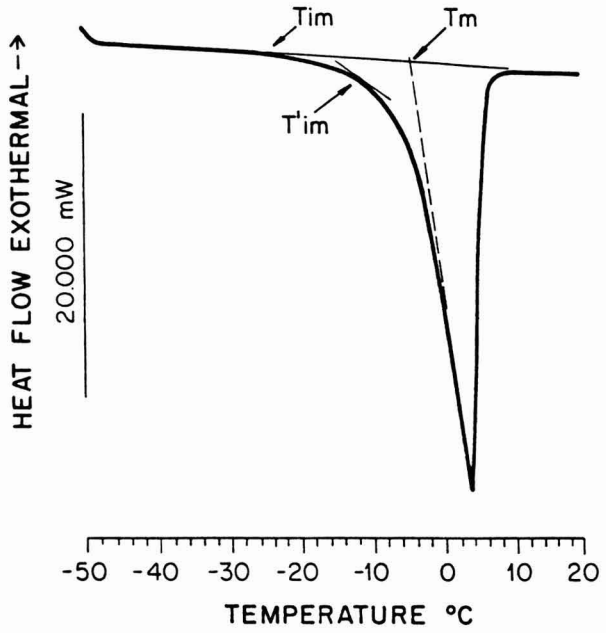

FIG. 3. DSC HEATING THERMOGRAM FOR FRESH STRAWBERRY

presented in Table 2. Results were classified according to strawberry cultivar and seed contents.

Based on Guegov's chart (1981) T_i for strawberry is between -0.5°C and -1.6°C for E values from 4%–10% respectively. Equation (5) yields $T_i = -0.52$°C for E = 5% w/w.

Using the n_{wo} values of tested cultivars in Eq. (6), T_i is -0.006°C for Florida and -0.33°C for Tioga.

T_i values in Table 1, determined from freezable water measured by DSC are lower than the predicted T_i by Eqs. (5) and (6), which take in account total water content.

T_i, also called the cryoscopic temperature, can not be read well in food cooling runs, because supercooling, even though undetected, is never avoided. T_i signals the start of water crystallization, which depends essentially on the solute concentration in the solution, but the solvent in the solution is not the total water of the food.

T_{sc} values from DSC curves were about -13°C which are lower than T_{sc} cited by Guegov (1981), which ranged from -5°C to -10°C for plant tissues, from -5°C to -6.5°C for fruits and -11°C for experimental freezing of grapes, using different cooling methods. T_{sc} were always lower than the almost constant initial freezing temperature of -1.8°C found in TA measurement.

TABLE 1.
CHARACTERISTIC TEMPERATURES FOR FREEZING PROCESS OBTAINED FROM DSC RUNS

Temperature	Strawberry Sample			
C	Florida		Tioga	
	only pulp	whole	only pulp	whole
T_i	-0.82	-0.83	-1.35	-1.41
T_{sc}	-13.25±1.3	-12.2±2	-15.6±1.1	-12.4±2.4

Since T_{sc}, signals the beginning of water solidification in the actual process, differences in T_{sc} can be attributed to different methods of cooling assessment, such as the imposed cooling rates, thermocouple sensitivity and location of the temperature measuring device, as already indicated by Fennema *et al.* (1973).

T_{cs} is associated with the precipitation of ice at the lowest eutectic temperature. Guegov (1981) established that supercooling and metastable equilibria are always present; therefore T_{cs} is not a unique value, being in the range of $-20°C$ to $-45°C$ for various food products, where as differences in T_{im} do not exceed a few degrees for fruits. A value of about $-45°C$ for T_{cs} obtained from DSC results, which is in the range cited by Guegov.

The freezing peak temperatures, T_{pc} is determined by the DSC equipment by automatically calculating the maximum heat evolution. Because of supercooling during DSC cooling tests was rather large, T_{pc} was not reliable or significant and the values obtained are not reported.

TABLE 2.
TEMPERATURES OBTAINED FROM DSC HEATING RUNS

Temperature	Strawberry Sample			
C	Florida		Tioga	
	only pulp	whole	only pulp	whole
T_{im}	-20.3±1.6	-22.8±2.3	-22.3±1.8	-23.2±2.4
T'_{im}	-9.6±1.1	-9.6±0.6	-12.0±1.0	-11.1±1.9
T_m	-2.8±0.3	-2.8±0.3	-4.1±0.5	-4.0±0.5
T_{ph}	-0.4	-0.5	-1.3	-1.5

T_{im} represents the melting temperature of ice in contact with the solution with the highest possible concentration, it represents the lowest eutectic temperature. It is generally accepted that superheating does not occur in heating determinations. The values given as T_{im} in Table 2, determined through DSC experiences after the curve and the base line were clearly separated are -20.3°C and -23.2°C. They are somewhat higher than those published by Guegov (1981) and Roos (1987), in the range of -30°C to -41.8°C $\pm$ 1.5°C.

T'_{im} was found by determining the first great change in the slope of the DSC curve after leaving the base line. The method was used to evaluate the molten fraction reported by Rey (1960) and cited by Roos (1986). The values reported in Table 2, -9.6°C and -12.0°C are dependent on strawberry cultivar. These T'_{im} are also higher than values given by Guegov (1981) and Roos (1987), which range from -15°C to -22.8°C.

T_m clearly signals the melting of ice crystals, which produces most evolution of heat. It is easily determined from thawing DSC curves. The value reported by Roos (1987) for strawberry cultivar Senga Sengana was T_m $-3.5° \pm 0.3$°C which is between the values calculated for the Florida cultivar -2.8°C, and Tioga cultivar -4.0°C, showing that different values are obtained for different varieties.

T_{ph} is also given by the DSC equipment in heating runs. T_{ph} values were close (less than 0.4°C higher) to the observed temperatures where the effective heat capacity was maximum.

CONCLUSIONS

DSC is useful for evaluating thermal properties in foods. It can provide characteristic product temperatures with a reliable accuracy. Some precautions have to be taken when T_{im} is measured because it characterized a phase change involving small quantities of heat, T'_{im} can be measured more accurately, and T_m can be determined even faster and more readily.

T_i can not be detected in DSC thermograms but it can be calculated by a proposed alternative method using only DSC data.

Characteristic temperatures for two cultivar were in the range cited for fruits and strawberries.

Characteristic temperature values change with the cultivar because changes in water content. From comparative results, characteristic temperatures were a few degrees lower for strawberry cultivar with less water.

Characteristic temperatures for samples with and without seeds were similar.

NOMENCLATURE

C_e = effective heat capacity (J/gC)
C_f = heat capacity in fully frozen state (J/gC)

DH = generalized enthalpy change (J/g)
DH_f = enthalpy change from DSC experimental curve integrated from -40°C to 10°C (J/g)
DH_o = latent heat of fusion of ice at the normal freezing point (J/g)
DH_w = total enthalpy change of pure water between -40° to 10°C (J/g)
n_w = freezable water
T = temperature (°C)
T_{cs} = complete solidification temperature (°C)
T_i = initial freezing point (°C)
T_o = water freezing point (°C)
T_{im} = minimum temperature of incipient melting (°C)
T'_{im} = maximum temperature of incipient melting (°C)
T_m = onset of melting temperature (°C)
T_{pc} = peak temperature in freezing (°C)
T_{ph} = peak temperature in thawing (°C)
T_{sc} = temperature of supercooling (°C)

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EFFECT OF LOW TEMPERATURE AND MODIFIED ATMOSPHERE STORAGE ON SUGAR ACCUMULATION IN POTATOES (*Solanum tuberosum*)

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ABSTRACT

The patterns of sugar accumulation in Onaway, Norchip and ND860-2 potato tubers were determined during storage at 9°C in air, and at 3°C in air and in modified atmospheres of 1000 ppm ethylene in air and 3% oxygen. Storage at 9°C was associated with essentially no sugar accumulation for all tubers evaluated. The accumulation of hexose (glucose and fructose) during 3°C storage temporally followed an increase in sucrose. The 1000 ppm ethylene atmosphere caused higher levels of sucrose to accumulate in Onaway and Norchip tubers, but delayed hexose accumulation in these tubers compared to storage in air. Storage at 3°C in low oxygen delayed, reduced and had no effect on sucrose accumulation in Onaway, ND860-2 and Norchip tubers, respectively, compared to storage in air. However, marked reductions in hexose accumulation were observed in ND860-2 and Norchip tubers stored at 3°C in low oxygen.

INTRODUCTION

A long-standing problem associated with the extended cold storage of potato tubers destined for processing is their tendency to accumulate sugars. Sugar accumulation results from starch breakdown (Isherwood 1976) and is associated with an undesirable darkening of processed products such as potato chips (Shallenberger *et al.* 1959; Burton 1969). The excessive darkening is believed to result primarily from the Maillard reaction (Mazza 1983; Fuller and Hughes 1984). Due to the seasonal nature of the production of potatoes, there is a need to develop means for extended low temperature storage of raw material for year-

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round processing. The ability to process potato stock into acceptable products directly from extended cold storage would also minimize the need for sprout inhibitors and reduce losses of solids due to decreased respiration (Coffin *et al.* 1987).

Recent efforts of potato breeding programs have yielded selections that are more resistant to low temperature sugar accumulation than some of the standard potato cultivars used for processing into chips (Johansen 1985, 1987). One such strain that is promising for use as a "cold-temperature chipper" (CTC) is the ND860-2 selection (Johansen 1987). This selection accumulates lower levels of sugar than conventional processing cultivars and this trait appears to be stable (Coffin *et al.* 1987). However, the physiological feature that imparts this behavior during low temperature storage of this selection remains unknown. Sowokinos (1987) suggested that strains suitable for processing directly from low temperature storage have a low glucose forming potential (GFP).

The purpose of this study was to evaluate the effects of low temperature and modified atmosphere storage on the patterns of sugar accumulation in a non-chipping cultivar (Onaway), a conventional chipping cultivar (Norchip) and the CTC selection, ND860-2. A 1000 ppm ethylene in air atmosphere was selected due to the differential effect that ethylene has on the retention of chipping quality in stored Monona and Kennebec tubers (Haard 1971). The other atmosphere featured a low oxygen (3%) content. This was selected since it appears to attentuate sugar accumulation during short term (4–5 weeks) exposure of tubers to low temperatures (Harkett 1971; Sherman and Ewing 1983) and it represents a treatment that is technologically feasible.

MATERIALS AND METHODS

Potatoes

Onaway and Norchip seed stock was obtained from local suppliers. ND860-2 seed stock was obtained from Dr. R. Johansen of North Dakota State University, Fargo, ND. All tubers were cultivated using standard commercial practices at the Arlington Experimental Farms of the University of Wisconsin. Tubers were hand-harvested about 2 weeks after foliar senescence, rinsed, air-dried and sorted for uniformity and absence of defects. Tubers were conditioned ("cured" or allowed to suberize) for 2 weeks at 20°C and 90–95% R.H. prior to storage.

Storage

Tubers were randomly divided into 4 lots and stored at 9°C in air or at 3°C in air and in modified atmospheres of 1000 ppm ethylene in air or 3% oxygen (balance of nitrogen) at 90–95% R.H. The modified atmospheres were generated

after placing tubers in 6 gallon Nalgene containers. The 1000 ppm ethylene modified atmosphere was generated by placing a calculated amount of ethephon (2-chloroethyl phosphonic acid, Sigma Chemical Co.) in a test tube suspended in the container and adding sodium hydroxide immediately prior to sealing. The 3% oxygen modified atmosphere was generated by flushing a sealed container containing the tubers with nitrogen until a residual level of 3% oxygen was obtained. These atmospheres were regenerated on a weekly basis. Verification of the levels of ethylene and oxygen was obtained by gas chromatographic analysis (GC, see below). Storage of tubers in air at 3 and 9°C was in unsealed containers. Reconditioning of tubers stored at 3°C was always done in air at 15°C for 2–3 weeks, regardless of the storage atmosphere.

Analytical

At each sampling interval a 100 g sample excised perpendicular to the axial plane from the center of 3–4 tubers was passed through a juice extractor (Braun, Model MP 32). The collected extract was diluted with 2 volumes of methanol. The diluted extract was centrifuged at 12,000 g for 15 min, filtered through Whatman #1 paper and a portion of the extract passed through an Alumina A Sep-Pak cartridge (Waters Associates). Sucrose, glucose and fructose were measured in the prepared extracts with an enzymatic test kit (Boehringer Mannheim Biochemicals). The average coefficient of determination observed for these studies was 22% and 16% for sucrose and hexose (glucose plus fructose) analysis, respectively.

Oxygen was quantified using an Aerograph Autoprep GC (Model A-700, Alltech Associates) fitted with a thermal conductivity detector. Ethylene was analyzed on a Shimadzu GC (Model 9AM) equipped with a flame ionization detector. Quantification of these gases was determined relative to external standards.

RESULTS

Sugar Accumulation

Sugar accumulation was measured as increases in sucrose and reducing sugars, the primary sugars that accumulate during low temperature storage of tubers (Isherwood 1976). We are reporting reducing sugar content as the sum of glucose and fructose (hexose) since they were present at roughly 1:1 ratios during this study, similar to the findings of others (Fuller and Hughes 1984).

Onaway Tubers. Sucrose accumulated to maximal values of 4 mg/g fresh weight (gfw) in Onaway tubers within 1 week of storage at 3°C in air (Fig. 1a). During extended storage, sucrose levels declined in association with an increase

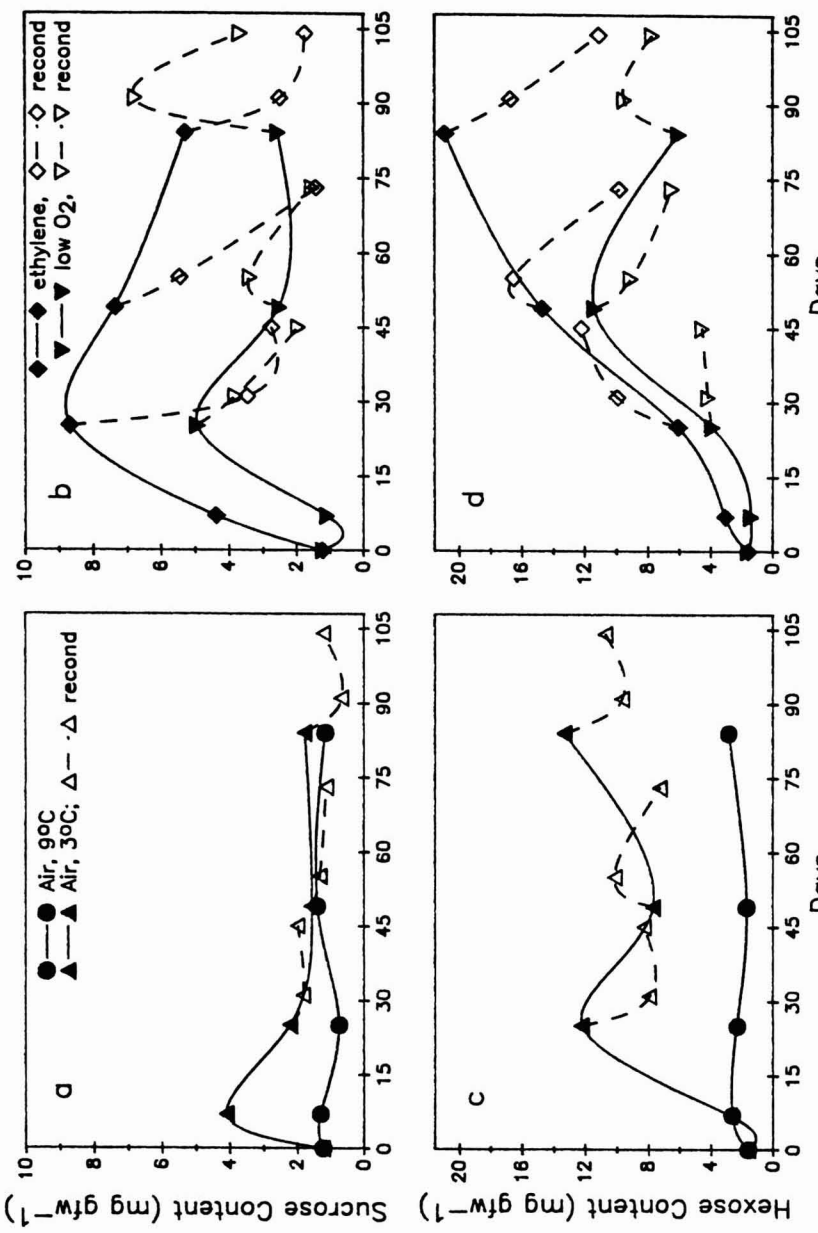

FIG. 1. SUCROSE AND HEXOSE ACCUMULATION IN STORED ONAWAY TUBERS
Solid lines and symbols represent changes taking place during storage.
Open symbols and dashed lines represent changes taking place during reconditioning (recond), initiated after about 3, 7 and 12 weeks of storage.

in hexose content to a maximum of about 12 mg/gfw by 4 weeks (Fig. 1c). Reconditioning after storage at 3°C in air had little effect on residual sucrose and hexose content. Tubers stored at 9°C in air accumulated little sugar.

Onaway tubers stored at 3°C in 1000 ppm ethylene accumulated sucrose (Fig. 1b) to maximal levels about 2-fold higher than those stored in air. Storage of these tubers at 3°C in the low oxygen atmosphere delayed the accumulation of sucrose compared to storage in air. Storage of Onaway tubers in both modified atmospheres slightly delayed the accumulation of hexose (Fig. 1d) compared to storage in air. Maximal hexose accumulation was similar (12 mg/gfw) for tubers stored at 3°C in air or low oxygen, whereas those stored in 1000 ppm ethylene accumulated the most hexose (20 mg/gfw). Reconditioning after storage in both modified atmospheres resulted in sucrose and hexose contents similar to those observed in tubers reconditioned after storage at 3°C in air.

Norchip Tubers. Storage of Norchip tubers at 3°C in air resulted in a maximal accumulation of sucrose (14 mg/gfw) within 3 weeks (Fig. 2a). During storage beyond 3 weeks, sucrose content decreased whereas hexose continued to accumulate to maximal levels (14 mg/gfw) by 7–8 weeks (Fig. 2c). Reconditioning after storage at 3°C in air led to decreases in sucrose and hexose content to about 2 and 2–4 mg/gfw, respectively. Tubers stored at 9°C in air accumulated little sugar.

The pattern of sucrose accumulation in tubers stored at 3°C in 1000 ppm ethylene (Fig. 2b) was similar to that observed in air. However, those stored in the ethylene atmosphere consistently maintained higher levels of sucrose. Storage of tubers in the 1000 ppm ethylene atmosphere also delayed the accumulation of hexose (Fig. 2d). Storage of Norchip tubers at 3°C in the low oxygen atmosphere caused sucrose to accumulate during the entire storage period, reaching a maximum of about 19 mg/gfw after 12 weeks (Fig. 2b). However, this atmosphere markedly reduced the rate and extent of hexose accumulation (Fig. 2d) compared to storage at 3°C in air or 1000 ppm ethylene. Reconditioning after storage in both modified atmospheres led to changes in sucrose and hexose content to levels similar to those observed after reconditioning following storage in air.

ND860-2 Tubers. ND860-2 tubers stored at 3°C in air accumulated sucrose to maximal levels of 6 mg/gfw after 3 weeks (Fig. 3a). Hexose accumulation commenced after 2–3 weeks of storage (Fig. 3c) and was associated with a decline in the sucrose content. Reconditioning after storage at 3°C in air decreased the residual sucrose and hexose content to 1–2 and 2–4 mg/gfw, respectively. Tubers stored at 9°C in air accumulated little sugar.

The patterns of sucrose and hexose accumulation for ND860-2 tubers stored at 3°C in 1000 ppm ethylene (Fig. 3b,d) were similar to those observed for tubers stored in air. In contrast, storage of ND860-2 tubers at 3°C in the low

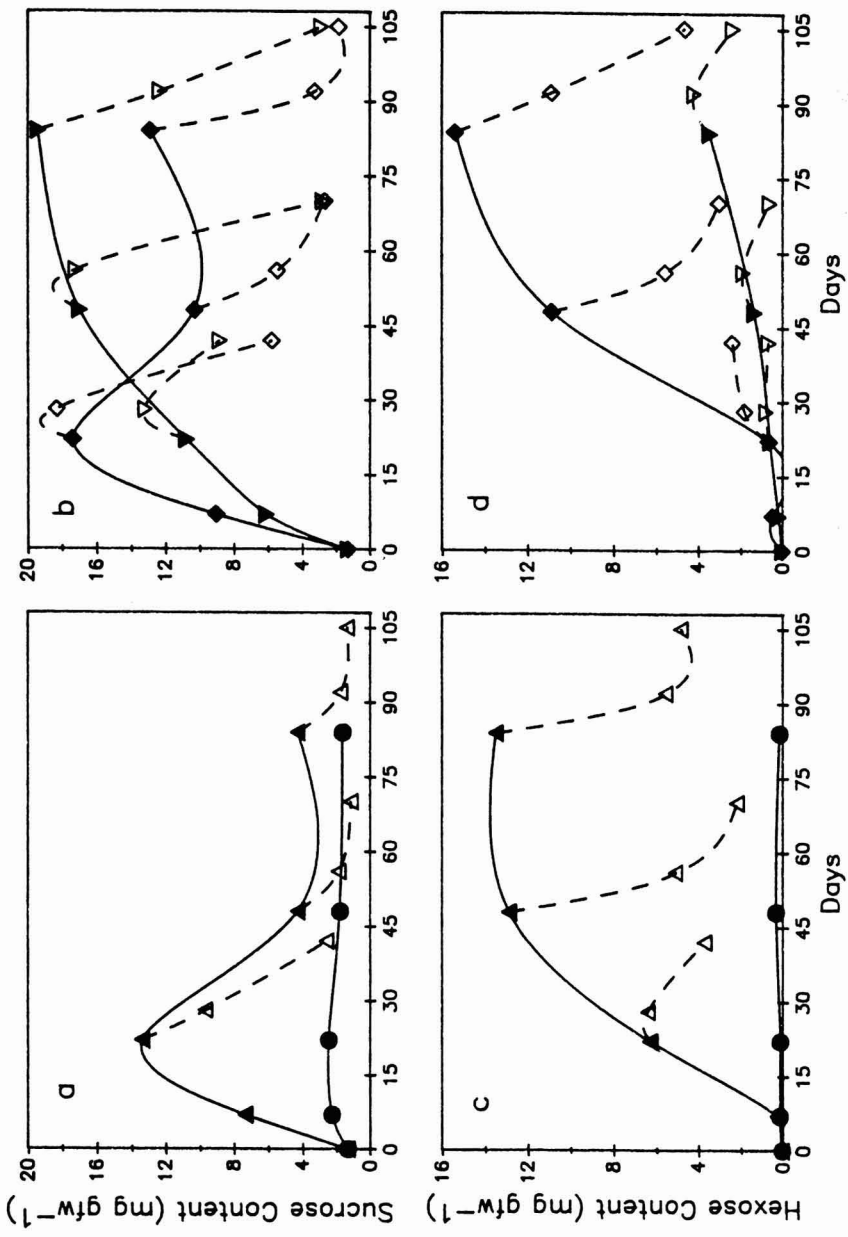

FIG. 2. SUCROSE AND HEXOSE ACCUMULATION IN STORED NORCHIP TUBERS.
Legend same as in Fig. 1.

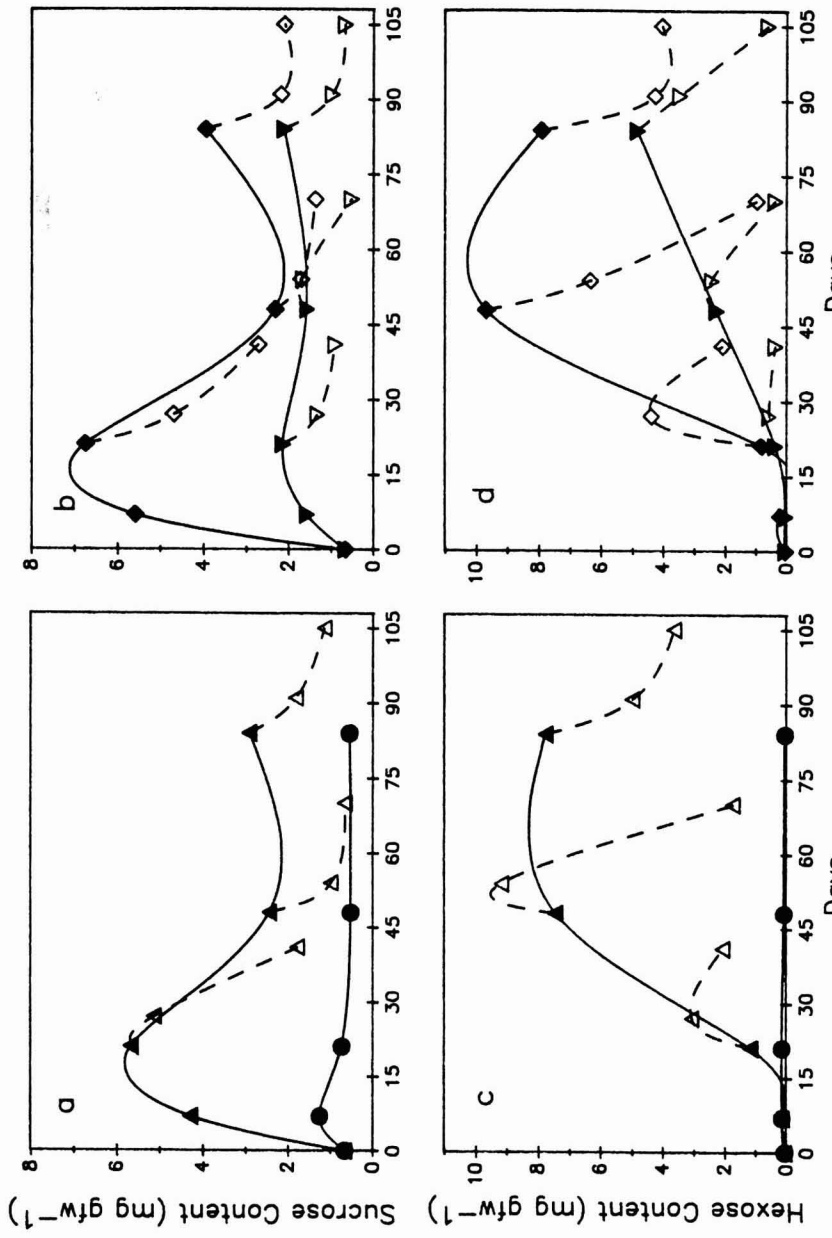

FIG. 3. SUCROSE AND HEXOSE ACCUMULATION IN STORED ND860-2 TUBERS
Legend same as in Fig. 1.

oxygen atmosphere reduced the extent of both sucrose (Fig. 3b) and hexose (Fig. 3d) accumulation. Reconditioning after storage in both modified atmospheres led to residual sucrose and hexose levels similar to those observed for tubers reconditioned after storage in air.

Relative Accumulation of Sucrose and Hexose

Sugar accumulation expressed as the mass fraction of hexose sugars (MFHS) relative to the total sugar content (sucrose plus glucose and fructose) is shown in Fig. 4. High MFHS values (0.5) were initially observed for Onaway tubers, and these values reached 0.8 within 4 weeks of storage in air at 3°C (Fig. 4a). Similarly high MFHS values (0.8) were observed for Onaway tubers stored at 3°C in 1000 ppm ethylene and low oxygen after 7 and 12 weeks, respectively (Fig. 4b).

Initial MFHS values were about 0.1 for both Norchip (Fig. 4c) and ND860-2 (Fig. 4e) tubers. MFHS values reached 0.8 by 7 weeks of storage of Norchip tubers at 3°C in air, whereas these values remained below 0.4 when tubers were stored at 9°C in air (Fig. 4c). The 1000 ppm ethylene atmosphere limited the increase in MFHS values to 0.6 (by 7 weeks) and the low oxygen atmosphere caused the MFHS values to remain below 0.2 for 12 weeks (Fig. 4d). MFHS values for ND860-2 tubers stored at 3°C in air (Fig. 4e) or 1000 ppm ethylene (Fig. 4f) reached 0.8 after 7 weeks. The low oxygen atmosphere delayed the increase in MFHS to 0.8 until about 12 weeks (Fig. 4f). MFHS values for ND860-2 tubers stored at 9°C in air remained below 0.2 (Fig. 4e).

DISCUSSION

The patterns of sugar accumulation during 3°C storage were evaluated for three cultivars/selection of tubers of differing suitability for processing (chipping) stock. Onaway is a non-chipper, Norchip is a chipping variety, and the ND860-2 selection has been identified as a cold-temperature chipper (CTC) (Johansen 1987; Coffin *et al.* 1987). Sowokinos (1987) suggested that the glucose-forming potential (GFP) of potatoes during cold stress is a better indicator of suitability for processing than is the potential to accumulate sucrose. However, sucrose accumulation is also important since it appears to be converted to reducing sugars (hexoses) during extended cold stress (Isherwood 1976), and sucrose itself may have a role in nonenzymic browning during high temperature processing (Shallenberger *et al.* 1959).

We assessed the GFP of these tuber cultivars/selection during cold storage in two ways: the amount of hexose accumulated, and the relative level of hexose and total sugar (hexose plus sucrose) accumulated (MFHS values). During storage in air at 3°C, Norchip and Onaway tubers accumulated up to 12–14 mg

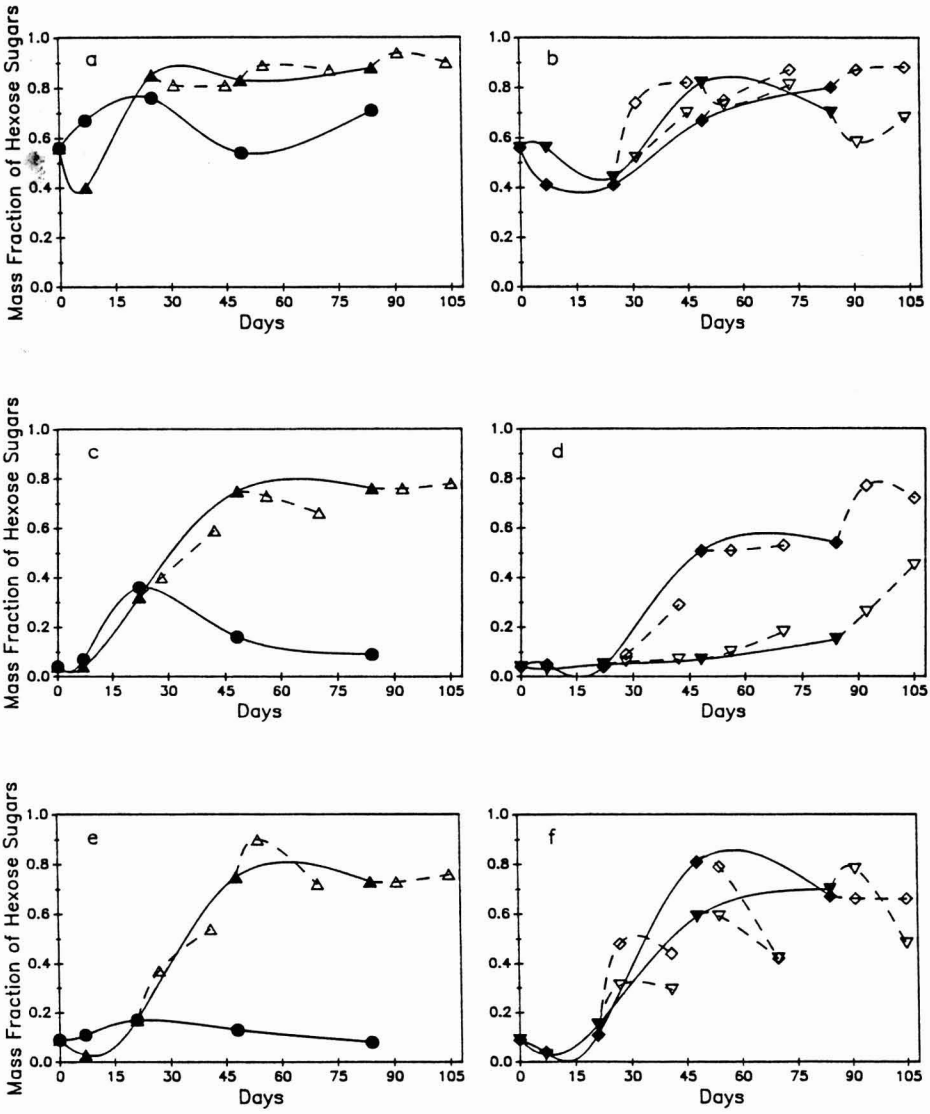

FIG. 4. MASS FRACTION OF HEXOSE: TOTAL SUGARS IN STORED TUBERS
(a,b) Onaway; (c,d) Norchip; (e,f) ND860-2. Legend same as in Fig. 1.

hexose/gfw, whereas ND860-2 tubers accumulated a maximum of 8 mg hexose/gfw. This study confirms an earlier one (Coffin *et al.* 1987) that ND860-2 tubers (a CTC selection) have a lower GFP than conventional processing cultivars. However, the maximum proportion of hexose accumulation under these storage conditions was similar for all three tubers, with MFHS values reaching about

0.8. Thus, in terms of the relative accumulation of hexose and sucrose, these tubers have similar "hexose-forming potentials" during cold storage. Therefore, the tendency of tubers to accumulate sucrose may be important in determining the amount of hexose that accumulates during cold storage.

During the initial period of 3°C storage in air, sucrose accumulated and reached maximum levels sooner in the Onaway tubers than in the Norchip and ND860-2 tubers. In all tubers, hexose accumulation during cold storage proceeded at a rapid rate only after sucrose had accumulated to levels several times higher than those observed immediately prior to cold storage. This implies that hexose accumulation is principally derived from sucrose, as Isherwood (1976) has suggested. This relationship is also supported by our analysis of MFHS values; these values initially decrease and/or remain below 0.1 during storage of all tubers at 3°C in air or in the modified atmospheres. We have also found this trend to hold for Russet Burbank and Norgold tubers stored at 3°C under these same conditions (Parkin and Schwobe 1990). Therefore, two strategies that could potentially limit hexose accumulation in cold stored tubers are to prevent sucrose accumulation or the conversion of sucrose to hexose.

The 1000 ppm ethylene atmosphere had little effect on the trend of sucrose accumulation, except that higher levels of sucrose were maintained in Onaway and Norchip tubers during extended cold storage compared to storage in air. For these tubers, the presence of 1000 ppm ethylene also delayed the initial rise in hexose content by only a week. However, the maximum total sugar accumulation in Onaway and Norchip tubers was about 35% and 85% greater, respectively, in the 1000 ppm ethylene atmosphere than in air at 3°C. Thus, there appeared to be little practical benefit of the ethylene atmosphere. The effect(s) of ethylene may be concentration- and/or cultivar-dependent. Haard (1971) noted that a brief exposure to 10 ppm ethylene prior to cold storage improved the retention of light chip color in Kennebec tubers, but not in Monona tubers. We have also reported that a modified atmosphere of 1000 ppm ethylene was not very effective in maintaining chip color in Norgold and Russet Burbank tubers stored at 3°C (Parkin and Schwobe 1990).

The effect of the low oxygen atmosphere was cultivar/selection-dependent. This treatment delayed sucrose and hexose accumulation in Onaway tubers and greatly limited sucrose and hexose accumulation in ND860-2 tubers. In Norchip tubers high sucrose levels were maintained in the low oxygen atmosphere, and hexose accumulation was attenuated. Thus, in the ND860-2 tubers, total sugar accumulation was inhibited, whereas in the Norchip tubers, the conversion of sucrose to hexose appeared to be impeded. In Russet Burbank and Norgold tubers, this low oxygen atmosphere reduced the extent of sucrose accumulation and the rate of sucrose conversion to hexose (Parkin and Schwobe 1990), similar to our findings here for the ND860-2 selection. A reduction in the GFP for

Monona, Kennebec, Majestic and Norchip tubers during short-term storage (4–5 weeks) at low temperature in reduced oxygen environments has been reported (Harkett 1971; Sherman and Ewing 1983). Collectively, these studies showed that a low oxygen atmosphere is beneficial in attenuating hexose accumulation in 7 of the 8 cultivars/selection evaluated to date.

A limitation of the low oxygen modified atmosphere was the development of blackheart in some tubers, particularly Norchip. This is known to result from anoxic conditions existing during storage of tubers (Sherman and Ewing 1983; Kader *et al.* 1989).

CONCLUSIONS

Our results showed that storage of tubers at 3°C in a modified atmosphere composed of low oxygen appeared to have some benefits compared to storage in air. Further studies are required to establish the optimum conditions in a low oxygen atmosphere before the feasibility of such a practice can be evaluated.

ACKNOWLEDGMENTS

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