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Fermentation of lady godiva (Cucurbita pepo) pumpkin flesh to produce ethanol

William M. Johnson

To the Graduate Council:

I am submitting herewith a thesis written by William M. Johnson entitled "Fermentation of lady godiva (Cucurbita pepo) pumpkin flesh to produce ethanol." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

H. O. Jaynes, Major Professor

We have read this thesis and recommend its acceptance:

F. A. Draughon, J. R. Mount

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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John R Mount

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

FERMENTATION OF LADY GODIVA (CUCURBITA PEPO) PUMPKIN FLESH TO PRODUCE ETHANOL

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

William M. Johnson

August 1981

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ABSTRACT

Flesh of Lady Godiva pumpkin was fermented to produce ethanol. Enzymes were used to hydrolyze fiber constituents to increase fermentable sugars. Analysis of the flesh showed 90.4% moisture and, on a dry matter basis, 11.3% protein, 1.8% lipid, 8.3% ash, 18.7% fiber (neutral detergent) and 60.0% nitrogen-free extract (NFE). Three cellulases were investigated and Cellulase Tv Concentrate showed the greatest increase in total sugars. Three strains of Saccharomyces yeast were used to ferment sugars to ethanol in mashes of pumpkin flesh with Cellulase Tv Concentrate added to break cellulose and increase sugars. Ethanol production was complete after 24 hours at 30^{o} C and no one strain of yeast was superior. Mean ethanol content was 0.86% in pumpkin mash diluted 1:1 (w/w) with water. Analysis of mashes before and after fermentation showed increases in moisture, protein and ash and a marked decrease in NFE.

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CHAPTER I

INTRODUCTION

The past several years have brought to the attention of everyone the fact that the world's energy supply, particularly petroleum products, is very limited. Continual price increases and spot shortages of imported oil have caused a massive effort to develop alternative energy sources. Although not a new development, one potential energy saving measure being considered is the use of gasohol, a mixture of approximately 10% absolute ethanol and 90% gasoline.

Recent research has shown that the seeds of the Lady Godiva pumpkin, naked seeded cultivar, are a potential source of good quality protein (7). Yields of this cultivar are high enough to allow production solely for the seeds (34). Such production would leave a large amount of pumpkin flesh as a byproduct. Although the flesh of Lady Godiva pumpkin can be utilized in such food products as pumpkin pies, breads and muffins (24), this can serve only as a very limited outlet. USDA data for pumpkins (44) shows that the flesh is high in sugar content and high in fiber content. Similar agricultural byproducts have been utilized to produce ethanol through yeast fermentations, many with combined hydrolysis of the cellulose present (38, 41).

These facts suggest that a potential use of the flesh byproduct of the Lady Godiva pumpkin would be the production of ethanol. The lack of reported research on the fermentation of pumpkin flesh to produce ethanol brought about this study to investigate the feasibility of

combined enzymatic hydrolysis of the cellulose present in Lady Godiva pumpkin flesh with yeast fermentation to produce ethanol.

CHAPTER II

REVIEW OF LITERATURE

I. PUMPKIN: HISTORY AND USES

The cucurbit family, Cucurbitaceae, includes all pumpkins and squashes. Included also are watermelons, cucumbers, muskmelons and gourds. Both pumpkin and squash varieties are found in the species Cucurbita pepo, Cucurbita muschata and Cucurbita maxima. Therefore, no clear botanical distinction exists between squashes and pumpkins (37).

The word pumpkin is generally thought of as a culinary term and as such is difficult to define. Whitaker and Bohn (45) stated that "pumpkin" was derived from Latin to indicate the gourd-like fruit of a cucurbitaceous vine utilized when ripe as forage, as a table vegetable or in pies.

Research indicates that pumpkins and squashes are of American origin. Erwin (15) was able to identify fragments of C. pepo and C. muschata from the cliff dwellers' ruins of southwest America and also from the basket makers, who are the oldest recorded agricultural people of North America.

Past usage of pumpkins has been somewhat limited. Pumpkins are usually thought of as the jack-o'-lantern so familiar at Halloween, and this is indeed a major commercial outlet for pumpkin. Pumpkin flesh is used in preparing such food products as pumpkin pies, pumpkin bread, cookies and puddings. In 1979, 2,877,000 cases of canned pumpkin and squash were produced along with 23,667,000 pounds of frozen pumpkin and

squash (2). Pumpkins are also used as livestock feed. These original uses of pumpkin flesh produce large amounts of seeds as byproducts. In certain Latin American countries and in Asia these seeds are roasted and sold in markets (45). Such uses of seeds are becoming more popular in this country with the increased interest in "natural" type products. Much interest has been shown in another use of pumpkin seeds as a possible oilseed and protein source.

One major problem with this use is in removing the seed coat. However, certain "naked seeded" varieties of C. pepo L. have been developed which have only a thin membranous seed coat which is much easier to remove. These small fruited varieties of C. pepo show the greatest promise as oil producers because of the large numbers of fruits per plant, with relatively large quantities of seeds (45). In 1972, the USDA released C. pepo L., Lady Godiva cultivar, and stated that yields were high enough to allow production solely for the seeds (34).

Recently a great deal of research has been done at The University of Tennessee on the utilization of the seeds of this Lady Godiva cultivar. Bhatia (7) found that these pumpkin seeds constitute a good source of protein and that the protein is of good quality, containing all the essential amino acids in fairly appropriate amounts. Devine (13) researched the functional properties of protein from Lady Godiva pumpkin seeds and found properties similar to those of certain protein concentrates.

The results of these experiments show the potential for a marketable product from the seeds of the Lady Godiva cultivar. If such a market is developed, a large amount of pumpkin flesh will be made available as a byproduct. Research by Jaynes and Gray (24) showed that the

flesh of Lady Godiva cultivar can be successfully utilized in conventional food products such as pumpkin pies, cakes, breads and cookies. Such usage is enhanced by the addition of spices and other flavoring ingredients. These products could serve as a limited outlet for the large amount of pumpkin flesh produced. Many of these products are thought of as somewhat novelty items and, even though processed pumpkin products are available year round, items such as pumpkin pies are consumed almost exclusively during the holiday seasons of Thanksgiving and Christmas. An alternative use would be the fermentation of pumpkin flesh to produce ethanol for fuel use. If successful this could prove to be a very timely and profitable process.

II. COMPOSITION AND UTILIZATION OF AGRICULTURAL BYPRODUCTS

Recently much attention has been given to the utilization of cellulosic materials as a potential energy source. While the supply of oil and petroleum products is limited, the supply of cellulosic materials appears to be unlimited. According to Sitton, et al.,(38) the usable agricultural residues in the United States total about 300 million tons per year, while Tsao, (42) estimated that 1,010 million tons of cellulosic wastes are available each year. The largest source of these cellulosic residues appears to be agricultural byproducts.

One of the largest sources of cellulose is from corn stover, which is estimated by Sitton, et al.,(38) to represent about one half the total from agricultural wastes. The processing of fruits and vegetables produces a huge amount of unused wastes, including seeds, peels, leaves, husks and juice (12). Some of these wastes are utilized in

animal feed but most are just wastes and as such must be disposed of or used in some way.

Detroy and Hesseltine (12) stated that the major components in agricultural residues are the structural cell-wall polysaccharides, mainly cellulose and hemicellulose. These represent the most plentiful renewable resources produced by green plants, often representing 45-70% of the weight of the dried plant. Lignin is also found in the cell wall construction of agricultural byproducts but usually in lower concentrations. Detroy and Hesseltine (12) found lignin to comprise from 3% to 15% of many dried plant residues. Lignin is the material which binds cellulose into fibers to give integrity and rigidity. Lignin is a polyphenolic material which forms a three dimensional seal around the cellulose. This seal forms a physical barrier which makes hydrolysis of cellulose difficult (23). The lignin content of fibrous plants seems to increase with age (12).

Hemicellulose is found in conjunction with cellulose and lignin in agricultural wastes. As described by Tsao (42), hemicellulose molecules consist of polymers of pentose sugars (xylose and arabinose), hexoses (mainly mannose) and some sugar acids. Hydrolysis of hemicellulose would obviously yield high concentrations of pentose sugars and slight amounts of mannose. Mannose is fermentable by alcohol producing yeasts while pentose sugars are not readily fermented, but may be utilized by certain fermenting strains (35). Often two sugar streams are made, one of pentoses and one of hexoses, and used appropriately (16).

Cellulose and starch can both be thought of as polymers of glucose. Starch is the storage carbohydrate of higher plants. It is made

up of the compounds amylose and amylopectin. Amylose is actually glucose molecules connected by an α-1,4 linkage while amylopectin consists of amylose units linked to each other by α-1,6 linkages. Cellulose, like amylose, is made up of glucose molecules; however, these molecules are linked by a β-1,4 linkage.

III. HYDROLYSIS OF CELLULOSE

The enzymatic and chemical hydrolysis of the α-1,4 linkage of amylose and the α-1,6 linkage of amylopectin are well known and well documented (1,11,40). Amylolytic enzymes are readily available and widely used in the fermentation industries.

Tsao (42) stated that the β-1,4 linkage of cellulose is no more difficult to break than the α-1,4 linkage of starch, from a process engineering standpoint. Difficulty in cellulose hydrolysis is due to two main reasons; first, the previously discussed lignin seal around the cellulose and second, the crystalline structure of cellulose.

Cellulose chains loop, forming folded chains. This looping gives rise to the crystalline structure of cellulose due to strong hydrogen bonding (23, 36, 42). Less ordered or frayed cellulose is termed amorphous. This amorphous portion is much easier to hydrolyze than the crystalline cellulose. Tsao (42) stated that pure cellulose is generally 15% amorphous and 85% crystalline.

Cellulose can be broken down to its glucose components by acid or enzymatic hydrolysis. Each method has its own advantages and disadvantages. These methods are compared by Ladish (27), Ryu and Mandels (36), and Spano (41). Acid hydrolysis requires the use of expensive corrosionproof equipment. Acid also causes decomposition of the

resulting sugars. Impurities in the substrate react with the acid resulting in unwanted byproducts. Acids are initially very expensive and must be recovered to make the process economical. The resulting sugars are not fermentable in the acid and must be separated. Some acid hydrolysis procedures require elevated temperatures, resulting in lowered sugar yields.

Enzymatic hydrolysis yields fairly pure glucose of constant composition. The enzymes are substrate specific and do not react with impurities or other materials present. The enzymatic hydrolysis reaction takes place under moderate conditions, thus increasing yields of glucose. Also due to the high moisture content, most agricultural residues are more suitable for biological processing (10). The major disadvantage of enzymatic hydrolysis is the expense of the enzymes. However, as demand increases and more research is done, the cost may decrease.

A problem in enzymatic hydrolysis of cellulose is the physical barrier of the lignin shield and the crystallinity. Many forms of pretreatment have been suggested to overcome these obstacles to help increase the glucose yield from cellulose wastes. Physical and chemical methods have been tried which include ball milling, hammer milling, steaming, treating with alkali and treating with solvent. These treatments add substantially to the costs and energy requirements of cellulose hydrolysis and therefore are used only when absolutely necessary. These pretreatments are outlined and discussed by Ghose and Ghosh (18), Kelsey and Shufizadeh (25), Ladish (27) and Nystrom and Andren (33).

The exact mechanism of hydrolysis of cellulose by enzymes has been, and is presently being, extensively researched. It is generally

agreed that such hydrolysis proceeds by a multienzyme system. Many researchers have tried to separate this complex and identify individual components. Flickinger (16), Ryu and Mandels (36), and Spano (41), suggest C_1 and C_x components to help understand the action of the cellulose complex. The C_1 complex has not been fully explained, but is classified as a prehydrolytic enzyme. This enzyme is an endoglucanase specific for crystalline parts of cellulose which acts as a prehydrolytic component and makes the cellulose susceptible to the C_x complex. The C_x complex consists of both exo- and endo-β-glucanases. These enzymes work synergistically and rapidly hydrolyze amorphous cellulose producing cellobiose and glucose. In general endo-glucanases hydrolyze one cellulose molecule into two smaller molecules, while exo-glucanases remove one glucose unit from the nonreducing end of the cellulose as outlined by Ghose and Ghosh (18). The term cellobiohydrolase is often used in describing these enzymes. Cellobiohydrolase has been described by Ghose and Ghosh (18), Flickinger (16) and Klyosov and Rabinowitch (26) as being very similar to exo-glucanase except that cellobiohydrolase specifically removes one cellobiose unit from the cellulose chain.

The third major component of the cellulase complex is cellobiase or β-glucosidase. These enzymes are very similar except that cellobiase is specific to hydrolysis of cellobiose while β-glucosidase can hydrolyze dextrins larger than cellobiose with a β-1,4 linkage (18). The terms cellobiase and β-glucosidase have been used interchangeably. According to Montenecourt and Eveleigh (31) and Ryu and Mandels (36), many different forms of each of these enzymes have been identified and as many as twelve cellulase components have been isolated.

An additional problem with cellulose hydrolysis by enzymes is that of end product inhibition. It is well known that end products, glucose and cellobiose, accumulate during saccharification and inhibit further action of the cellulase complex. This has been discussed by many authors including Flickinger (16), Herr (21), Montenecourt and Eveleigh (31) and Ryu and Mandels (36). Ryu and Mandels (36) stated that the inhibition is competitive while Montenecourt and Eveleigh (31) suggest that the inhibition is noncompetitive. In either case, end product inhibition can be a major problem in enzymatic hydrolysis of cellulose. Herr (21) stated that under proper conditions the continuous removal of glucose from the reaction could increase saccharification significantly. Herr (21) also found that incomplete hydrolysis could be attributed to increasing crystallinity of the cellulose as the reaction proceeds as well as to enzyme deactivation.

Many organisms are capable of producing cellulase. Some of these mentioned by Herr et al. (22), Murao et al. (32), and Nystrom and Andren (33) are various forms of fungi including Aspergillus niger, Lenzites trabea, Mycothecium vervucaria, Trichoderma viride and Fusarium sp. Cooney et al. (10), Ghose and Ghosh (18) and Mandels and Andreotti (29) describe certain anerobic bacteria found in the digestive tract of rumanants as being able to hydrolyze cellulose also.

Cellulase is an inducible enzyme and is usually obtained by controlled fermentation of the selected organism on a cellulose medium as described by Mandels and Andreotti (29) and Gallo et al. (17). Commercially, cellulase production has been practically limited to Trichoderma and Aspergillus species. Both species are well known cellulase producers. Research suggests that Aspergillus produces cellulase

with high β-glucosidase activity but low endo- and exo-glucanase activities while Trichoderma species produce cellulase with high glucanase activities but may be slightly deficient in β-glucosidase. It generally is agreed that the cellulase from Trichoderma is the best and most economical available today, Gallo et al. (17), Herr (21) and Ryu and Mandels (36).

Currently a great deal of research is being done to increase cellulase production and β-glucosidase activity by developing mutant strains of Trichoderma species (1). According to Gallo et al.(17) and Montenecourt and Eveleigh (31) mutant strains of Trichoderma ressei show great promise as cellulase producers. Herr et al. (22) describes the wild strain of Trichoderma viride, ITCC-1433, as producing large amounts of a well balanced and active cellulase rich in β-glucosidase.

IV. ETHANOL PRODUCTION AND USES

The conversion of sugars to ethanol can be accomplished by either chemical or biological means. Production of ethanol by yeast fermentation is a well known process. Extensive research has been done on this cycle, with Pasteur beginning research as early as 1872. Pasteur developed the theory that fermentation by yeast is a consequence of the life processes of nutrition, assimilation and growth when carried out under anaerobic conditions (30). Under such conditions certain yeasts are able to break down sugar (mainly glucose) to produce alcohol and carbon dioxide with the release of heat. This heat energy then is used for metabolism and growth. The general equation developed for the fermentation process is:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

It is generally recognized that about 51.1 percent of the sugar is converted to ethanol while 48.9 percent goes to carbon dioxide, on a weight basis (35). These are theoretical yields and in actual practice these exact values cannot be obtained. Actual yields normally amount to 90 to 95 percent of the theoretical values. The remaining 5 to 10 percent can be accounted for in the accumulation of byproducts (chiefly glycerol and succinic acid) and by the utilization of sugar for yeast growth and metabolism (35, 40).

Since the time of Pasteur, the fermentation cycle has been fully elucidated and has been found to be a complex biochemical pathway (9).

Maximum ethanol production varies according to yeast strain, substrate, fermentation conditions and other factors. The greatest ethanol concentration produced has been about 19% (v/v). This was under rigidly controlled conditions using a substrate of high sugar concentration with the addition of sugars as fermentation proceeded. An average yield would be considered to be about 11% to 17% ethanol from a substrate containing about 32% sugar (35). Ethanol yield per unit of sugar fermented will vary during the fermentation process. This yield will be higher during the later stages because intermediates formed during the early part of the cycle will then be converted to ethanol. Also the use of sugars for the growth of the yeast will begin to slow.

Ethanol accumulation is known to have an inhibitory effect upon fermentation (19). However, high levels of ethanol are necessary for inhibition to take place. For example, it has been shown that ethanol concentration of up to 5% (v/v) has little effect on most strains of alcohol producing yeasts (35).

Rose and Harrison (35) give the optimum conditions for fermentation as an initial pH of 4.5 to 5.5 with a temperature of 30^{o} to 40^{o} C. However, fermentations have been known to stop at temperatures above 32^{o} C. In practice the inoculation level of yeast is in the range of 0.1% to 1.0% based on the final volume of medium.

The major yeast for alcohol production is Saccharomyces cerevisiae. Many varieties of this yeast have been developed for use depending upon the fermentation conditions and type of product being made. For example, in wine manufacturing a yeast is needed which can withstand the high acidity of grape juice and which can tolerate ethanol levels greater than 10%. Saccharomyces cerevisiae var. ellipsoideus has been found suitable for such work (35). A major yeast for the brewing and distilled spirits industries is Saccharomyces carlsbergensis. This yeast also has been found to be a good alcohol producer and to be relatively alcohol tolerant.

Species of another yeast, Schizosaccharomyces, have been tried for alcohol production. These yeasts are known to ferment organic acids as well as sugars. Schizosaccharomyces species require a higher optimum temperature for fermentation than do the Saccharomyces species. It has been noted that Schizosaccharomyces ferment sugars about 50% slower than do Saccharomyces species (35).

The alcohol produced must be separated from the nonfermentable components, yeast cells and fermentation byproducts. This separation is usually accomplished through filtering and distillation.

The boiling point of ethanol is 78.5^{o} C. If a mixture of water and alcohol is boiled, the proportion of alcohol to water in the vapor is always greater than the proportion in the liquid (30). Condensation of

the vapor results in high concentrations of alcohol. To obtain the purest alcohol more than a single distillation column is required. Often a series of distillation columns or towers are used. Under proper operating conditions, up to 95% ethanol can be obtained from a single distillation column. Some type of drying agent must be employed to obtain 100% ethanol.

The use of alcohols is not a recent development. Ethanol has been known since early civilization, but its use was mainly as an intoxicant. According to Slesser and Lewis (40) alcohol came into use as an energy source in about 1830. Its use was mainly for lighting purposes, replacing the fish and whale oils used at the time. Ethanol was in turn replaced by kerosene.

During the infant stages of the internal combustion engine, alcohol was used as a source of fuel (40). In Brazil, alcohol has been combined with other fuels and used as automobile fuel since 1930 (28). At about that time one company in Brazil was selling fuel consisting of 75% alcohol and 25% ether (40). Petroleum, however, was found to be a cheaper fuel source and it also contained more potential energy than the alcohol/petroleum mixtures. During both World Wars the supply of petroleum became limited and these alcohol/petroleum mixtures again came into use. Since that time the use of these fuel mixtures has been continued only in a few countries. Brazil has become a leader in gasohol production and use because Brazil has almost no domestic petroleum resources. Gasohol is a term used for the mixture of gasoline and anhydrous ethanol, usually 90% gasoline with 10% ethanol. In 1975 the Brazilian government started the Brazilian National Alcohol program. The goal of this program is ultimately to reduce Brazil's consumption

of imported petroleum by blending gasoline with agriculturally produced ethanol (80% gasoline to 20% ethanol) (28). It has been estimated that Brazil will be self-sufficient in alcohol production by 1984 (40).

In the United States the production of ethanol from agricultural products and the use of gasohol are on the rise. The continued increase in the price of imported crude oil and the threat of short supplies are two major reasons that these processes must be further developed.

CHAPTER III

MATERIALS AND METHODS

I. SOURCE AND PREPARATION OF RAW MATERIALS

Pumpkins, Lady Godiva cultivar of C. pepo, were grown on the University of Tennessee Plant and Soil Science Farm. These were harvested at maturity in late August and early September 1980.

After harvesting, the pumpkins were opened and the seeds and placental material removed. Seeds were washed, dried with forced air at 50° C, sealed in polyethylene bags and stored at 4° C for future use. Pumpkin flesh was cut into pieces approximately 5 cm square, washed in cold water and blanched for 10 minutes in boiling water. Pumpkin pieces were drained of excess water and approximately two kilograms were sealed in polyethylene bags and stored at -23° C until used.

II. PROXIMATE ANALYSIS

For proximate analysis on raw material, pieces of pumpkin were removed from randomly selected polyethylene bags. Three separate samplings were done on different days.

For each sample, pieces were thawed, drained of excess water and blended at 1:1 ratio (w/w) with distilled water for 2 minutes in a Waring blender to give a pourable mixture. These suspensions were lyophilized in a Virtis freeze dryer and compositional analysis (other than moisture) performed on the dry matter.

Proximate analysis was also done on all fermentation mixes before and after fermentation.

A. Moisture

Percentage moisture was determined on raw material by drying approximately five grams of pumpkin flesh to constant weight in a vacuum oven at 60° C and 300 torr, as outlined in AOAC method 7.003 (3). Three replications of triplicate samples were done.

Before and after fermentations, single large samples from each treatment and replicate were taken to dryness in the Virtis freeze dryer. Percentage moisture was calculated by weight loss.

B. Protein

AOAC method 7.015, the Kjeldahl procedure, was used to determine total nitrogen (3). Two gram samples were used for analysis. Percentage protein was calculated by multiplying total nitrogen x 6.25. For raw material, three replications were carried out using triplicate samples. Duplicate analyses were performed on each before and after fermentation sample.

C. Crude Lipid

Crude lipid was determined by petroleum ether extraction using the Goldfisch fat extraction apparatus as described in AOCS method AC3-44 (4). One and one-half gram samples were extracted with petroleum ether for six hours. Crude lipid percentage was calculated. Raw material analyses were done in triplicate on three samples. Before and after fermentation, duplicates from each sample were measured.

D. Ash

As outlined in AOAC method 7.009 (3), percentage ash was calculated by dry ashing one and one-half grams of sample in a muffle furnace

at 550° C for four hours. Three analyses were done on each of three samples for raw material. Analysis of before and after fermentations was done in duplicate.

E. Fiber

1. Neutral detergent fiber (NDF). One gram samples of dry pumpkin were analyzed for total fiber content using the NDF procedure of Georing and Van Soest (20). By subtracting the NDF from 100, an estimate of cell soluble material was determined. Ash insoluble in neutral detergent solution was determined by ashing the residue for 3 hours at 550° C. Three determinations were done on triplicate samples for raw material. Two analyses were done on each before and after fermentation sample. The NDF was the only fiber determination done on the before and after fermentation samples. Again these were done in duplicate.

2. Acid detergent fiber (ADF). The ADF procedure of Georing and Van Soest was used to determine lignocellulose content of dried pumpkin (20). Determinations were made using one gram samples. Triplicates were done on three samples of raw material.

3. Acid detergent lignin (ADL). ADL was determined as outlined by Goering and Van Soest using the previously determined ADF residue (20). Again, triplicates were done on three samples.

4. Cellulose and Hemicellulose. Cellulose content was determined by subtracting the acid detergent lignin from the acid detergent fiber. Hemicellulose was calculated by subtracting the acid detergent

fiber from the neutral detergent fiber. These calculations are outlined by Van Soest and McQueen (43).

F. Carbohydrates

1. Nitrogen-Free Extract (NFE). The total carbohydrate content (NFE) was determined by subtracting the percentages of moisture, protein, lipid, ash and fiber (NDF) from 100 percent. NFE was the only carbohydrate determination done on before and after fermentation samples.

2. Total soluble sugars. Soluble sugars were determined by using a colorimetric method as outlined by Dubois et al. (14), and Barnett and Tawab (5). A Shimadzu Model UV-190 double beam spectrophotometer was used. Three replications of triplicate samples were done on raw material using one gram samples.

3. Starch. Starch content was determined through extraction, hydrolysis and detection as glucose with Somogyi's reagent as outlined in AOAC method 3.120 (3). Triplicate analyses were done on three 0.8 gram samples of dry raw material.

III. ENZYME EVALUATION

A. Enzymes: Sources and Specifications

Three separate enzyme complexes were obtained for evaluation. All were received in dry powder form.

1. Cellulase C was obtained from Fermco Biochemics, Inc., Elk Grove Village, Illinois. The following information and specifications were provided by the manufacturer. Cellulase C is obtained from cultures of certain Aspergillus species. In addition to its cellulase

activity, several secondary activites are noted for Cellulase C. Important among these are β-glucosidase, β-glucosidase and the splitting of cellobiose.

2. Rohalase M-4 was also obtained from Fermco Biochemics. Information obtained from the manufacturer stated that Rohalase M-4 is an enzyme complex obtained from Aspergillus cultures. Main activities are amylase and cellulase with various glycosidases as accompanying enzymes.

3. Cellulase Tv concentrate was obtained from Miles Laboratories, Inc., Elkhart, Indiana. Manufacturer's specifications stated that Cellulase Tv concentrate is obtained from Trichoderma viride. In addition to its cellulase activity, this complex possesses significant glucanase, hemicellulase and pentosanase activities. Lower activities of α-amylase, lipase and protease were also specified.

B. Preparation and Sampling

The following procedure was used for each enzyme. Pumpkin pieces were taken from randomly selected polyethylene bags of the previously processed material. Pieces were thawed, weighed and mixed 1:1 (w/w) with distilled water then blended to give a pourable suspension. Two hundred grams of this thick slurry were placed in each of four 500 ml Erlenmeyer flasks. The pH of these mixtures was adjusted to 5.0 by the addition of 1N HCL. Flasks were then autoclaved at 121° C under 15 psi for 20 minutes.

Appropriate amounts of enzyme were weighed to give enzyme to substrate (cellulose) ratios of 0.2%, 0.4% and 0.8%. Each of these was made to a volume of 25 ml by mixing with 0.2M acetate buffer of pH 5.0.

Each enzyme solution was sterilized by passing through a Nalgene filter sterilization unit with 0.2 micron pore size. The sterilized enzyme solutions were added aseptically to individual flasks containing the pumpkin slurry. Each flask was marked appropriately 0.2%, 0.4% and 0.8%. The fourth flask was used as a control to which 25 ml of filter sterilized acetate buffer was added.

After addition of the enzyme solutions, these flasks were placed in an American Optical water bath shaker. Water temperature was set at 30^{o} C and the shaker adjusted to approximately 100 cycles/minute.

Single 2.5g samples from each mixture were aseptically pipeted into small sample bottles at 12-hour intervals over 48 hours. Sample bottles were placed in a boiling water bath for 5 minutes to inactivate the enzyme. These were then frozen until all samples for that particular evaluation had been collected. This entire procedure was replicated once.

C. Measurement of Enzyme Effectiveness

The degree of hydrolysis of cellulose and other carbohydrates by these enzyme solutions was measured by the increase in total sugars. The samples were thawed and prepared according to Barnett and Tawab (5). Total sugars were measured by the phenol-sulfuric acid method of Dubois et al. (14), using the Shimadzu UV-190 double beam spectrophotometer. Duplicate evaluations were done for each sample.

Standard curves were prepared for each evaluation as follows. A glucose solution containing 0.1g/liter was prepared. Aliquot portions were taken and diluted with distilled water to give concentrations between 20 mg/2ml and 200 ug/2ml glucose. Blanks were prepared using

2 ml distilled water. These standards were prepared for absorbance readings according to the Dubois procedure (14).

The Shimadzu UV-190 spectrophotometer was used to determine the wavelength of maximum absorbance. This was found to be 490 nm by first running a scan on standard glucose solution and then comparing with absorbance readings on individual samples.

IV. FERMENTATION PROCEDURE

A. Evaluation of Yeasts

Four strains of ethanol-producing yeasts were obtained.

1. A dried strain of Saccharomyces cerevisiae was obtained from Dr. B. J. Demott, Department of Food Technology and Science, The University of Tennessee. This yeast came originally from Bio-con, Lexington, Kentucky, as a special distillers' yeast.

Since the yeast was obtained in a dried form, it was necessary to prepare a pure, thriving culture. This was carried out as follows: About 0.5 grams of dry yeast was suspended in a sterile dilution blank. A loop of this suspension was aseptically placed into each of three screw cap tubes containing 10 ml of yeast-dextrose broth. Tubes were incubated at 32^{o} C. After 24 hours, tubes were gently shaken to disperse the cells throughout the media. A loop of the broth, from each tube, was aseptically streaked onto a mycophil agar plate. The plates were incubated at 32^{o} C for 24 hours. At that time a single colony of yeast growth from each plate was picked to yeast dextrose broth tubes and incubated as before. After this incubation the complete streaking and incubation processes were repeated. Single colonies were then

transferred to mycophil agar slants and grown at 32° C for 24 hours. Slants were then maintained at 4° C until needed.

2. Pure cultures of the following yeast were obtained from Dr. J. O. Mundt, Department of Microbiology, The University of Tennessee.

a. Saccharomyces cerevisiae var. gentry

b. Saccharomyces carlsbergensis-ATCC 4228

c. Schizosaccharomyces octosporus-ATCC 855

Cells from each culture were grown on mycophil agar slants and maintained at 4° C.

Each yeast was evaluated for ethanol production in broth containing 5% glucose, 1% malt extract and 1% yeast extract (w/v). One-liter batches of this solution were placed in each of four individual 2-liter Florence flasks and the pH adjusted to 5.0 by the addition of 1N HCL. Flasks were closed with gauze covered cotton plugs and autoclaved for 20 minutes at 121° C.

An inoculum of each yeast was prepared by transferring a loop of growth from mycophil agar slants to tubes of yeast dextrose broth. Broth tubes were incubated for 24 hours at 32° C. Individual Florence flasks of fermentation media were then inoculated with 1% of the appropriate yeast-dextrose broth culture.

The Florence flasks were then closed with fermentation locks which had been sterilized by soaking for 10 minutes in a 50 ppm chlorine solution. Each fermentation lock was filled with sterile water to provide an air trap. Flasks were then placed in a 30° C water bath for fermentation.

Before sampling, each flask was gently swirled by hand to give a homogeneous mixture. Sampling consisted of aseptically removing

duplicate 5 ml portions from each flask at 12-hour intervals over 96 hours. Individual samples were sealed in sample bottles and frozen until all had been collected.

Ethanol production was measured by gas chromatography according to the procedure of Bouthilet et al. (8), using acetone as an internal standard. Specific conditions and modifications of this procedure are given in the following section.

B. Alcohol Determination

Prior to actual analysis of samples for ethanol content, a response ratio was determined. Standards were prepared containing absolute ethanol and pesticide grade acetone. The internal standard (acetone) percentage was maintained at 2%, while the ethanol percentage was varied incrementally by 0.75% from 0.5% through 3.50%. Duplicate solutions were prepared at each ethanol percentage level by mixing appropriate weights of acetone and ethanol and diluting to 100 grams with distilled water. Duplicate injections of 1 µl were made from each standard solution into a Shimadzu Mini-2 gas chromatograph equipped with a flame ionization detector, Dohrman recorder, and a Shimadzu Chromatopac E/A electronic calculating integrator. The column was 5 feet x 0.125 inch stainless steel packed with Porapak Q (100/120 mesh, Applied Science Labs). Specific conditions for the gas chromatograph were as follows:

Column temperature - 150° C

Injection/Detector temperature - 165° C

Attentuation - 64

Carrier gas (nitrogen) flow rate - 60 ml/minute

Hydrogen flow rate - 34 ml/minute

Air flow rate - 345 ml/minute

Acetone and ethanol peaks were distinguished by alternately injecting acetone/ethanol standard solutions and solutions containing only acetone or ethanol. Retention times for each component were matched. Response ratios were calculated for each injection using the formula: RR_x = rfx/rfs where rfx equals peak area of sample/sample concentration and rfs equals peak area of internal standard/concentration internal standard.

The previously collected samples from fermentation trials were then analyzed for ethanol content. After thawing, 0.1 ml of pesticide grade acetone was added to each 5 ml sample, giving 1.96% internal standard. Duplicate injections were made from each sample. Single injections were made from the 72 hour, 84 hour and 96 hour samples. Percent ethanol was calculated using the formula:

$C_x = (A_x/A_s) \times (C_S/RR_x)$ where A_x is the peak area of ethanol, A_x is the peak area of the internal standard, C_s is the concentration (%) of the internal standard and RR_x is the previosuly determined response ratio. These ethanol concentrations were then converted to a weight basis, using a method similar to that described in the next section. The three most active ethanol-producing yeasts were selected for use in fermentation of Lady Godiva pumpkin flesh.

C. Fermentation of Pumpkin Flesh

Pieces of pumpkin were removed from randomly selected bags of the frozen pumpkin. The pieces were then thawed, weighed and blended 1:1 (w/w) with distilled water in a Waring blender for approximately

2 minutes. A total of 2000 grams of this slurry was prepared. The pH of the slurry was adjusted to 5.0 by the addition of 1 N HCL. Five hundred grams of this suspension were removed, sealed in a polyethylene bag and frozen for later proximate analysis. The remaining 1500 grams were placed in a 2-liter Florence flask and closed with a gauze wrapped cotton plug. This procedure was done in triplicate (one flask prepared for fermentation by each yeast) for each of three replications. Closed flasks were then autoclaved for 20 minutes at 121^{o} C under 15 psi.

Inocula of the three selected strains of yeast was prepared in yeast dextrose broth and 1% of 24-hour cultures was added to the pumpkin preparation.

To provide additional fermentable sugars, filter-sterilized suspensions of Cellulase Tv concentrate were added to the pumpkin preparation at a level approximating 0.8% of the cellulose present.

After these additions, each flask was gently shaken by hand to effect mixing of all components. Flasks were fitted with a fermentation lock which had been sterilized and filled as described earlier, and flasks were incubated in a 30^{o} C water bath. Three replications of the fermentation procedure were done.

Duplicate samples were taken from each flask at 12-hour intervals over 96 hours. Sampling involved aseptically removing 15 ml of the fermentation medium using a sterile, disposable pipet. This 15 ml sample was vacuum filtered through No. 42 Whatman filter paper, yielding just over 5 ml of filtrate. Of this filtrate, 4.9 ml were removed and placed in sample bottles and tightly capped. The samples were frozen until each replication was completed. At the completion of each

fermentation, the contents of each flask were removed, sealed in a polyethylene bag and frozen for later proximate analysis.

At the completion of each replication, samples were thawed and 0.1 ml of pesticide grade acetone was added to give 2% internal standard. Duplicate injections from each sample were made into the Shimadzu Mini-2 gas chromatograph to determine ethanol concentration. Conditions for the gas chromatograph and calculations were as previously described.

Since the samples were prepared on a volume basis (4.9 ml sample plus 0.1 ml acetone), it was necessary to convert the calculated ethanol concentration to a weight basis, and also to adjust for the 2% dilution by the addition of acetone. This was done by increasing the calculated ethanol percentage by 2% and then multiplying this new value by 0.798, the density of ethanol at 15.5^{o} C. No correction for the ratioed densities of ethanol and acetone was made since they are almost equivalent. Ethanol density at 15.5^{o} C was chosen because samples had just been thawed prior to analysis. All ethanol concentrations were reported on a weight basis.

V. STATISTICAL ANALYSIS

All proximate analysis data for raw material were averaged and means and standard deviations reported. The data for gas chromatography response ratio, ethanol production and proximate analyses before and after fermentations were analyzed by analysis of variance using the Statistical Analysis System (SAS) (6) and The University of Tennessee Computer Center. Significance among ethanol means over time was measured by Tukey's Test (39).

CHAPTER IV

RESULTS AND DISCUSSIONS

I. CHEMICAL ANALYSES OF PUMPKIN FLESH OF LADY GODIVA CULTIVAR

A slurry of 1:1 (w/w) pumpkin flesh and distilled water was prepared for proximate analysis. Adjustments were made for this water addition in calculating the percentage moisture of the samples.

The proximate composition of Lady Godiva pumpkin is presented in Table 1. Data from the USDA (44) is presented for comparison purposes. It can be noted that these values are in close agreement with data presented from the USDA (44) which was for pumpkin in general.

The percentage of nitrogen-free extract (NFE) on a wet basis, 5.78%, is presented also. This is of particular interest since the actual fermentation of pumpkin flesh was done on a wet basis and the NFE contains the fermentable sugars.

Table 2 contains data from the analysis of types of carbohydrate present in Lady Godiva pumpkin flesh. Again, sugar content, as total soluble sugars, was given on a dry matter basis and a wet basis.

These results would indicate that Lady Godiva pumpkin flesh should be an adequate substitute for growth of ethanol-producing yeasts. When considering fermentation, several of these results are of particular interest. Since sugars (chiefly glucose) are the necessary substrate for production of ethanol, they are of primary interest. The nitrogen-free extract indicates a total sugars concentration of 5.78%, wet basis, while the total soluble sugars is 4.11%. The difference in these might be explained by the fact that nitrogen-free extract is

TABLE 1

COMPOSITION OF PUMPKIN FLESH

Component	Lady Godiva[a]	USDA Data[b]
	%	
Moisture	90.36 ± 0.50	91.6
Protein	11.34 ± 0.27	11.90
Ash	8.34 ± 0.12	9.52
Lipid	1.76 ± 0.10	1.19
Fiber (NDF)	18.67 ± 0.91	13.10[e]
Nitrogen-Free Extract[c]	59.89 ± 0.89	64.29
Nitrogen-Free Extract[d]	5.78 ± 0.30	

[a]Means of nine observations ± one standard deviation.

[b]Reported as grams per 100 grams edible portion - converted to dry matter basis for comparison.

[c]100 - (protein + ash + lipid + fiber); dry matter basis.

[d]100 - (moisture + protein + ash + lipid + fiber), wet basis.

[e]AOAC crude fiber.

TABLE 2

DISTRIBUTION OF CARBOHYDRATES IN LADY GODIVA PUMPKIN FLESH

Component	Concentration[a] %
Neutral Detergent Fiber (NDF)	18.67 ± 0.91
Acid Detergent Fiber (ADF)	12.77 ± 0.88
Acid Detergent Lignin (ADL)	0.96 ± 0.13
Cellulose[b]	11.80 ± 0.83
Hemicellulose[c]	5.90 ± 0.85
Total Soluble Sugars	42.63 ± 1.34
Total Soluble Sugars (WB)[d]	4.11 ± 0.13
Starch	1.05 ± 0.10

[a]Means of nine observations ± one standard deviation, dry matter basis.

[b]Cellulose = ADF - ADL.

[c]Hemicellulose = NDF - ADF.

[d]Calculated on a wet basis.

determined by difference, thus encompassing potential error from five separate experimental procedures, while the total soluble sugars is a single experimental process. Regardless of these differences, the sugar content of Lady Godiva pumpkin appears to be sufficient for yeast fermentation.

Important also are the percentages of cellulose and starch. Both of these components represent potential fermentable sugars, since both are long chain polymers of glucose. Many commercial enzymes are available which can hydrolyze the β-1,4 linkage of cellulose, the α-1,4 linkage of amylose and the α-1,6 linkage of amylopectin to increase the concentration of fermentable sugars.

The relatively high concentration of cellulose, 11.80% (dm), would suggest that enzymatic hydrolysis might possibly increase the fermentable sugar content of the substrate. The 1.05% (dm) starch content indicated that enzymatic hydrolysis of this component would not yield a substantial increase in fermentable sugars.

II. EVALUATION OF ENZYMES

Based on the results of proximate analysis of Lady Godiva pumpkin, it was decided to attempt to increase the concentration of fermentable sugars through enzymatic hydrolysis of the fiber present.

Three separate enzyme complexes were obtained, Cellulase C, Rohalase M-4 and Cellulase Tv Concentrate. These enzymes were added individually to suspensions of pumpkin flesh and water, 1:1 (w/w), at concentrations of 0.2%, 0.4% and 0.8% enzyme to cellulose ratios, based on manufacturers' recommendations. Enzymes were evaluated under standard conditions of pH 5.0 and in a 30^{o} C shaking water bath.

Effectiveness of enzymes was measured by determining the increase in total sugars over 48 hours according to the spectrophotometric procedure of Dubois et al. (14). Absorbances were measured on the 2 ml samples at 490 nm. Dilutions of a 0.01g/liter glucose standard solution were used to determine a linear standard curve before each evaluation. Straight line curves, which followed Beer's law, were obtained when plotting absorbance against concentration. A typical standard curve is shown in Figure 1. Through linear regression equations, ($y = mx + b$), of these curves, the concentrations of soluble sugars were determined per 2 ml sample and the total soluble sugars calculated.

Figure 2 shows the percentage of soluble sugars over time for two replicate evaluations of 0.2%, 0.4% and 0.8% Cellulase C in a pumpkin: water slurry. In replication one, Figure 2a, the greatest increase in soluble sugars was 1.17%. This was found after 48 hours with the 0.8% enzyme solution. In replication two, Figure 2b, an increase of 0.63% was found after 24 hours with the 0.8% enzyme solution.

Figure 3 illustrates similarly the increase in soluble sugars in pumpkin:water slurry over time with the use of Rohalase M-4 enzyme. Figure 3a shows that in replication one the greatest increase in soluble sugars was 1.33% after 36 hours using a 0.8% enzyme to cellulose ratio. For replication two, Figure 3b shows the greatest increase in soluble sugars to be 0.75%, again using the 0.8% enzyme solution after 36 hours.

Figure 4 shows the duplicate evaluations of Cellulase Tv Concentrate at three different concentrations. From Figure 4a, a 2.09% increase in soluble sugars can be noted after 24 hours using the 0.4% enzyme solution. Similarly, a 2.06% increase is found with the 0.8% solution. Figure 4b shows that in replication two, a 0.89% increase in

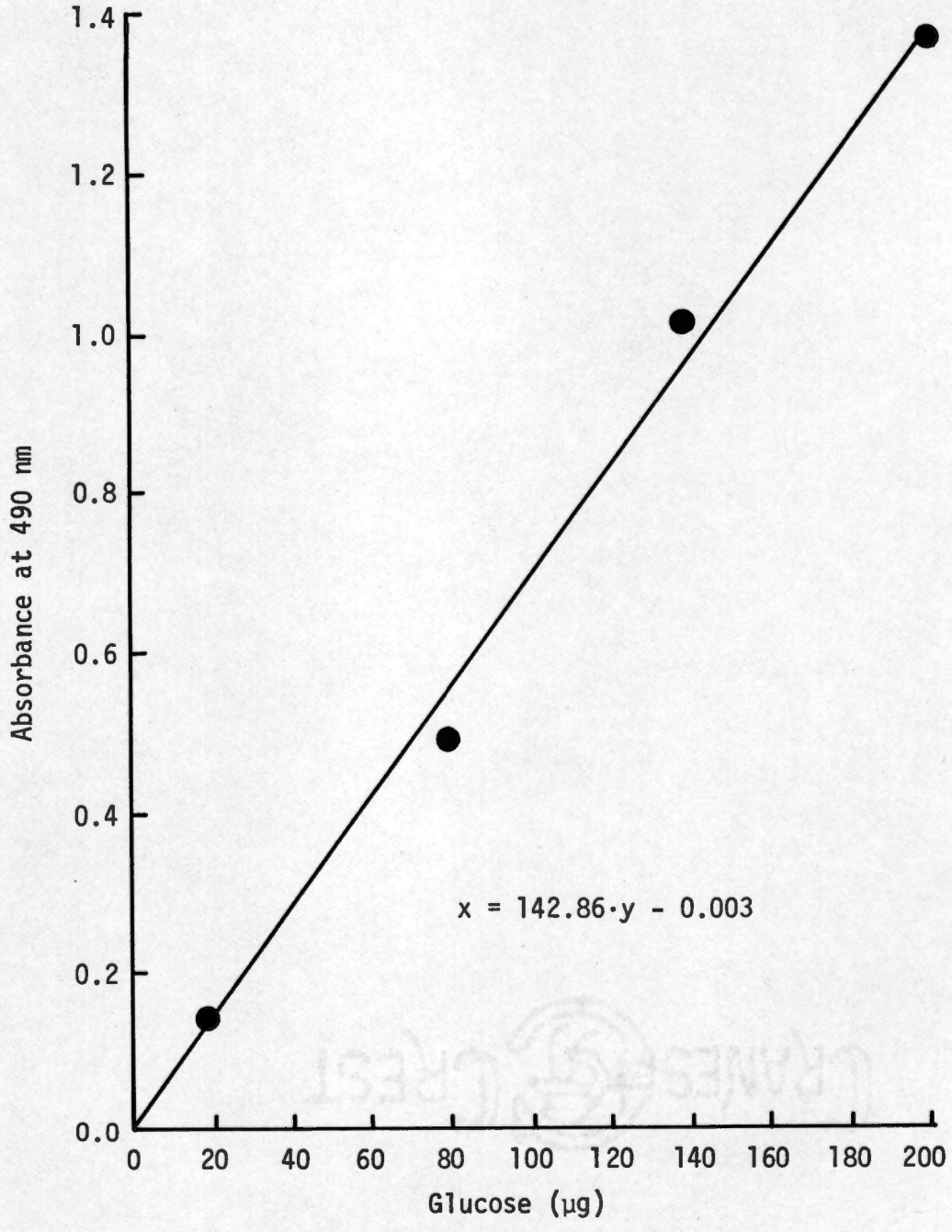

Figure 1. Typical Standard Curve of Absorbance Readings vs. Amounts of Glucose.

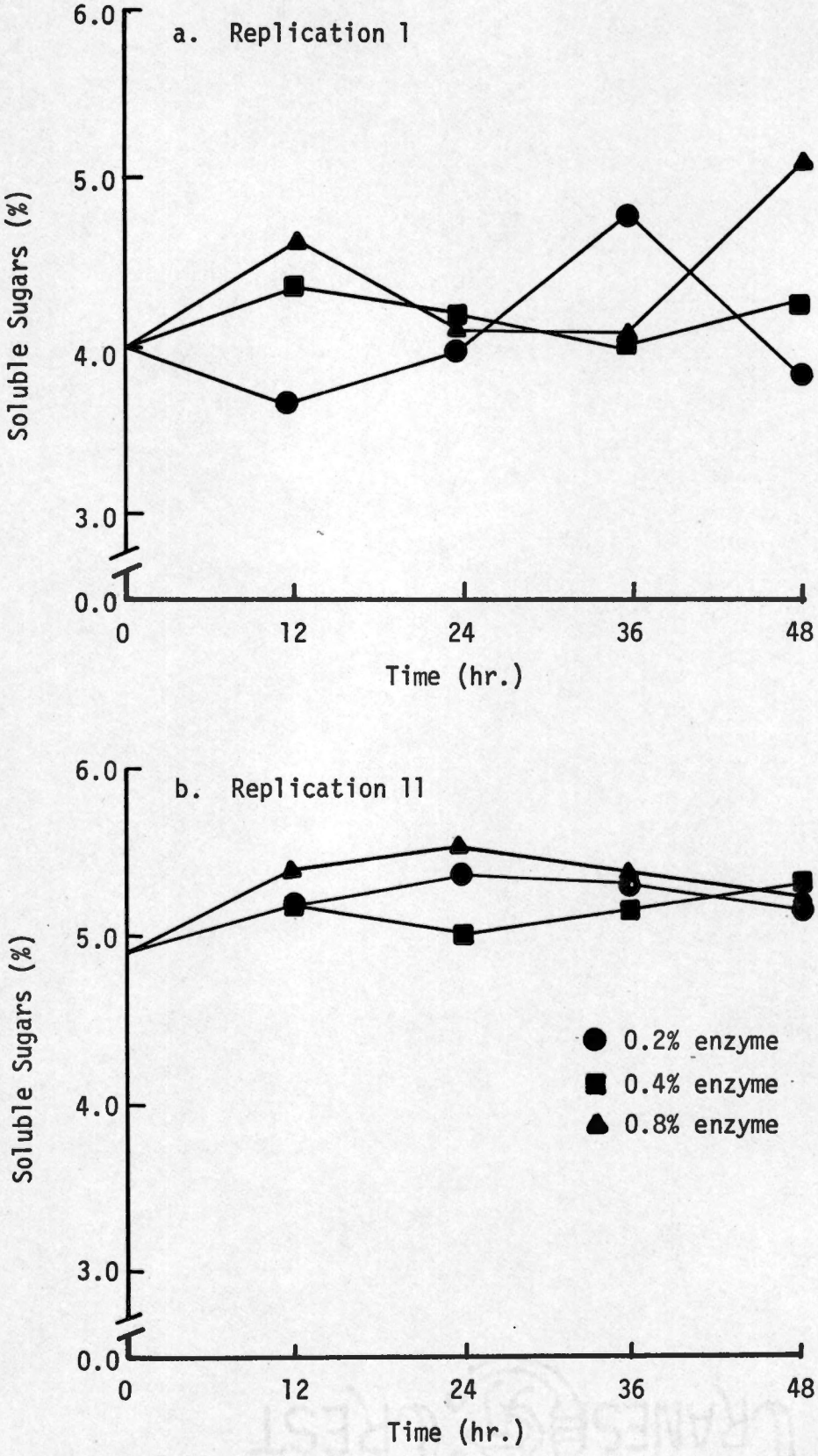

Figure 2. Soluble Sugars Over Time in Pumpkin: Water Slurry During Hydrolysis with Cellulase C.

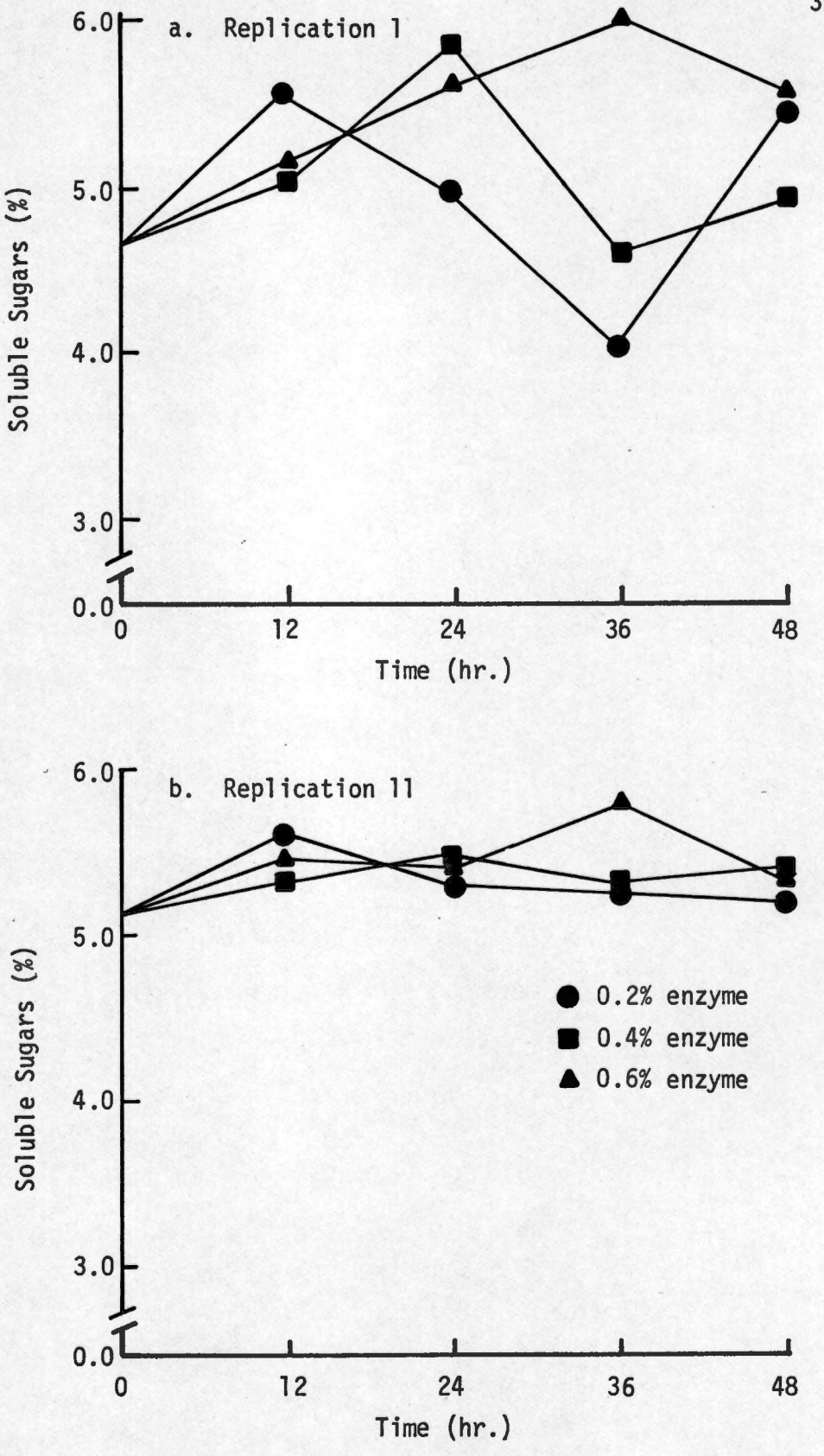

Figure 3. Soluble Sugars Over Time in Pumpkin: Water Slurry During Hydrolysis with Rohalase M-4.

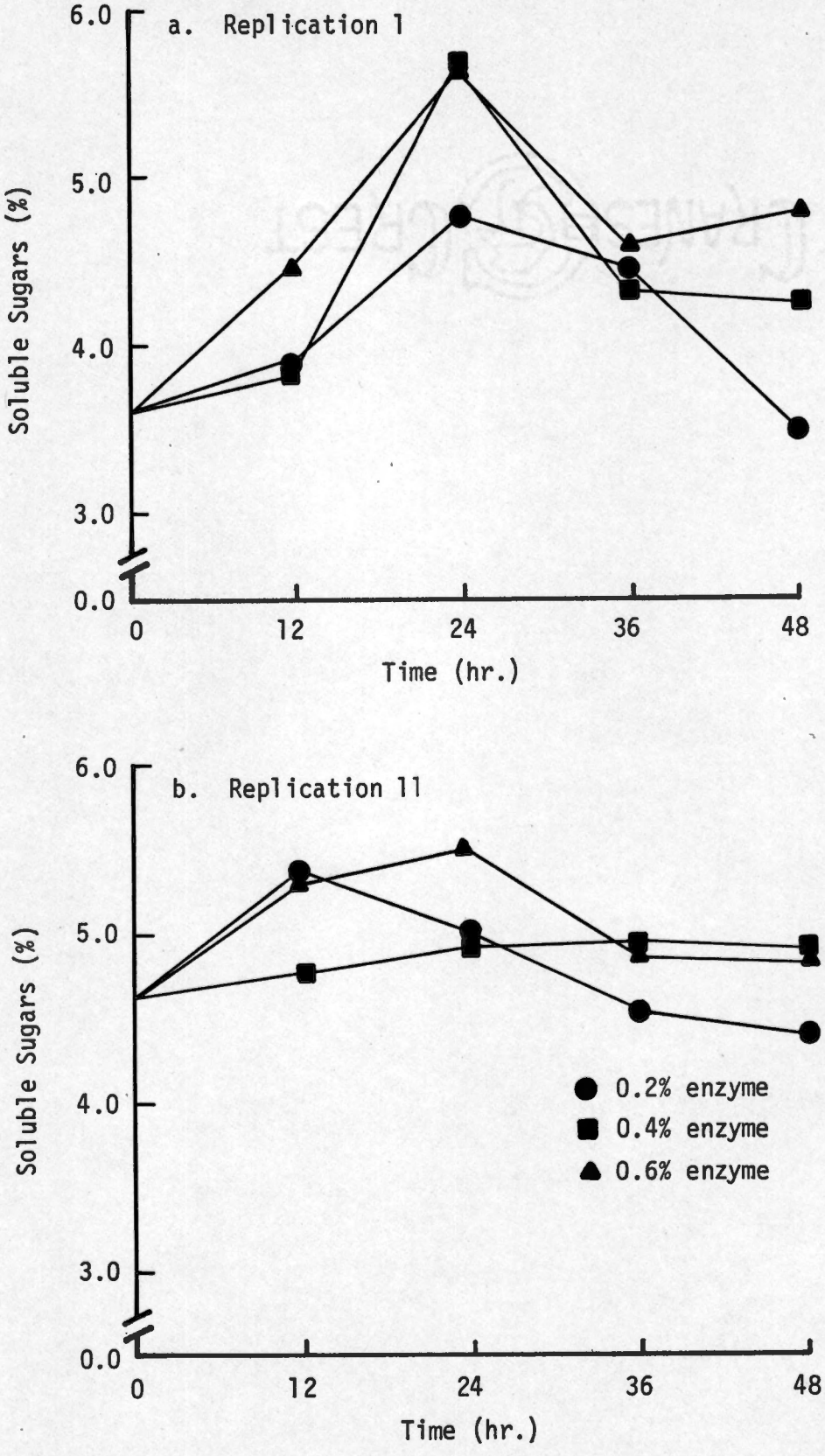

Figure 4. Soluble Sugars Over Time in Pumpkin: Water Slurry During Hydrolysis with Cellulase Tv Concentrate.

soluble sugars is obtained using the 0.8% enzyme solution after 24 hours. Table 3 lists the greatest increase in total soluble sugars in pumpkin: water slurry for each enzymatic evaluation.

These results demonstrate the difficulty of enzymatic hydrolysis of cellulose, particularly when using impure, complex substrates. Some variations in effectiveness would be expected between duplicate evaluations, but certainly not to this degree. Variations would be expected due to the actual differences in composition of the duplicate samples. Concentration of substrate (cellulose) would cause variations. Also variation in the lignin content of the pumpkin could inhibit the action of the cellulase enzymes. The lignin seal around the cellulose is a known obstacle to hydrolysis (23). Since no pretreatments, except autoclaving and blending, were employed to break this lignin seal, any lignin present would have an inhibitory effect.

The actual blending of each sample could have been of importance. Easily accessible cellulose is more susceptible to enzymatic hydrolysis (18, 25). Inadvertent differences in blending of samples could have caused differences in hydrolysis.

The inhibition of cellulase enzymes by end-products (glucose and cellobiose) accumulation is well documented (16, 21, 31). Therefore, it would be expected that each hydrolysis would reach a certain glucose concentration and maintain at that level. Also some glucose is initially present in the pumpkin and this could cause inhibition to a certain degree.

None of these facts could explain the decrease in glucose concentration of most samples after reaching a maximum increase. This would seem to suggest other possibilities like errors in sampling the thick pumpkin slurry.

TABLE 3

INCREASES IN TOTAL SUGARS OF LADY GODIVA PUMPKIN FLESH:WATER SUSPENSIONS AFTER HYDROLYSIS WITH VARIOUS ENZYME COMPLEXES

Enzyme Complex	Replication	Maximum Increase in Total Sugars (%)	Means
Cellulase C	1	1.17	0.9 ± 0.38
Cellulase C	2	0.63	
Rohalase M-4	1	1.33	1.03 ± 0.43
Rohalase M-4	2	0.72	
Cellulase Tv Concentrate	1	2.09	1.49 ± 0.85
Cellulase Tv Concentrate	2	0.89	

Due to the wide variations in duplicate analyses and the unexplained decreases in soluble sugar content within samples, no statistical analysis was done on these results. Cellulase Tv Concentrate was chosen for hydrolysis of cellulose present in Lady Godiva pumpkin flesh during fermentation. This was based on the higher mean increase in total soluble sugars and the fact that these maximums were reached early in the hydrolysis (24 hours). It was hoped that by reaching maximum hydrolysis early, any glucose produced would be fermented, thus avoiding end-product inhibition.

From the literature (17, 21) it was not unexpected that the cellulase from Trichoderma viride showed the greatest mean cellulose hydrolysis.

III. SELECTION OF YEAST STRAINS AND FERMENTATION OF LADY GODIVA PUMPKIN FLESH

Four yeast strains, Saccharomyces cerevisiae, Saccharomyces cerevisiae var. gentry, Saccharomyces carlsbergensis and Schizosaccharomyces octosporus, were evaluated for ethanol production using a broth containing 5% glucose, 1% yeast extract and 1% malt extract. Duplicate samples were removed from each fermentation flask at about 12-hour intervals over 96 hours and frozen until all samples had been collected. These samples were then analyzed for ethanol content by gas chromatography using the internal standard method of Bouthilet et al. (8).

Before this analysis was done a response ratio was calculated using ethanol and acetone (2%). The analysis of variance of the calculated response ratios is presented in Table 4. It can be noted that the response ratios are not significantly different at the 0.05 level.

TABLE 4

ANALYSIS OF VARIANCE OF RESPONSE RATIOS, ALCOHOL AND ACETONE (2%) BY GAS SOLID CHROMATOGRAPHY

Source	df	ms	f-ratio
Relative Conc.	4	0.0003964	1.42436^{ns}
Error	5	0.0002783	
Total	9		

^{ns}Not significant at 0.05 level.

Therefore, consistent instrumental response would be expected over a large range of ethanol concentrations within samples. As a result, the overall mean of these response ratios, 0.894, was used in all calculations for ethanol content by gas solid chromatography. Table 5 gives the response ratios from each standard solution by ethanol concentration, along with the overall mean.

The samples collected from the fermentation of 5% glucose broth by the four yeasts were then analyzed by gas solid chromatography. Figure 5 shows the mean ethanol produced by each strain over time. Saccharomyces cerevisiae and Saccharomyces cerevisiae var. gentry showed very similar ethanol-producing abilities, with both yeasts reaching a maximum ethanol level of about 2.76%. Saccharomyces carlsbergensis produced somewhat less ethanol, showing a maximum of 2.44% after 96 hours. The fourth yeast, Schizosaccharomyces octosporus, was obviously the poorest ethanol producer. This yeast reached a maximum ethanol production of 1.15% after 96 hours.

These results were not surprising based upon information from the literature. Rose and Harrison (35) explain that Schizosaccharomyces species ferment glucose at about half the speed of Saccharomyces species and also that Schizosaccharomyces species exhibit a higher optimum temperature for fermentation.

Based on these differences, Saccharomyces cerevisiae, Saccharomyces carlsbergensis and Saccharomyces cerevisiae var. gentry where chosen for fermentation of Lady Godiva pumpkin flesh.

Fermentations of Lady Godiva pumpkin flesh were carried out as outlined in Materials and Methods. Throughout this discussion Strain 1

TABLE 5

RESPONSE RATIOS, ALCOHOL AND ACETONE (2%) BY GAS SOLID CHROMATOGRAPHY

Ethanol Concentration %	Response Ratio[a]
0.5	0.895
0.5	0.868
1.25	0.891
1.25	0.900
2.00	0.908
2.00	0.907
2.75	0.886
2.75	0.930
3.50	0.875
3.50	0.881
Overall Mean	0.894

[a]Means of duplicate injections from each solution.

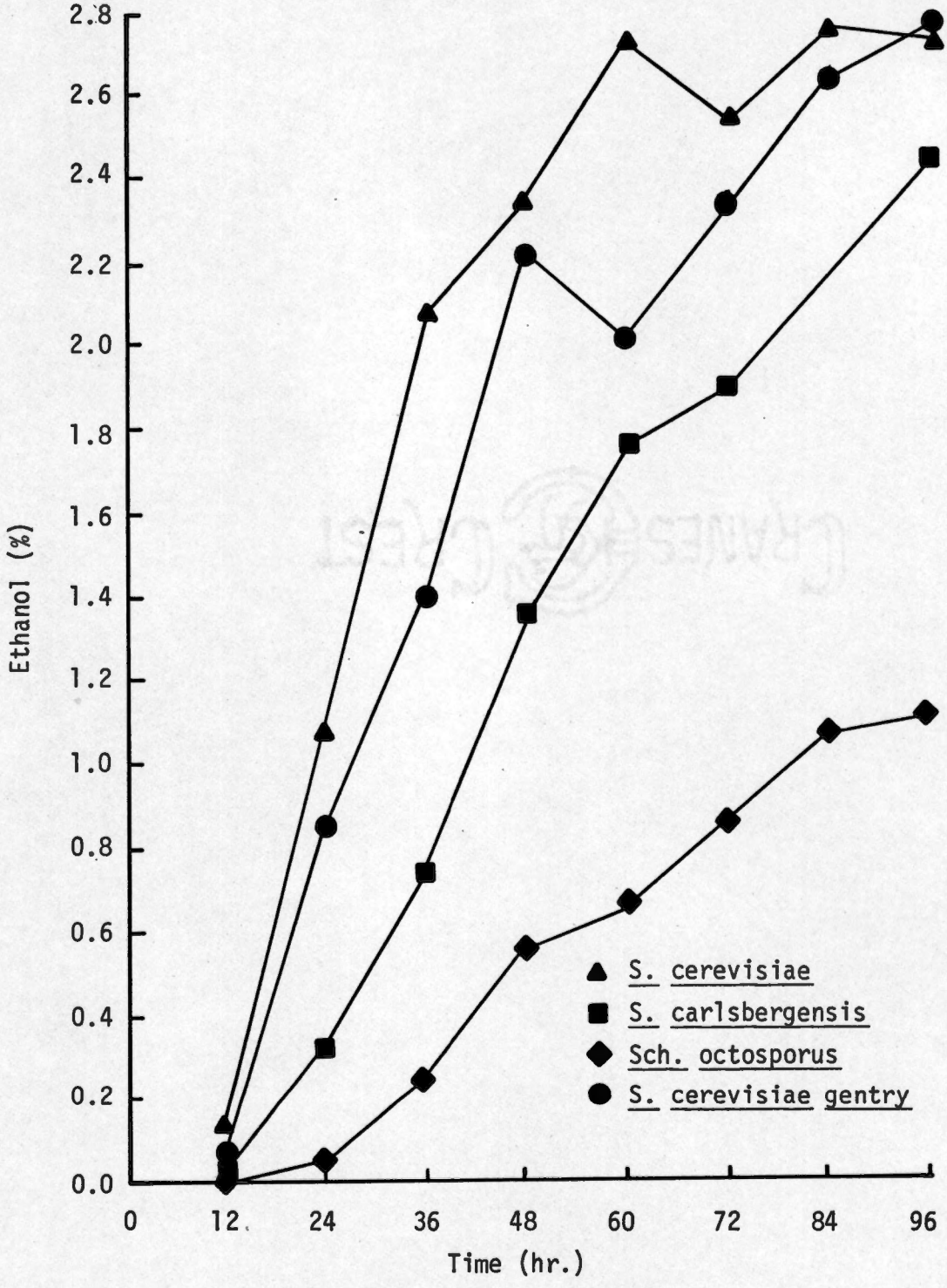

Figure 5. Ethanol Produced by Four Yeast Strains in 5% Glucose Broth.

represents Saccharomyces cerevisiae, Strain 2 represents Saccharomyces carlsbergensis and Strain 3 represents Saccharomyces cerevisiae var. gentry.

Table 6 presents the analysis of variance of ethanol production from three replications of fermentation of Lady Godiva pumpkin flesh by three yeast strains over time. It can be noted that neither yeast strain nor replication showed a significant difference at the 0.05 level. This would indicate that no one strain of yeast was found to produce more ethanol than another in the experiment. Also it suggests that overall, no significant difference was noted among replications.

It should be noted that the strain x replication interaction was significant at the 0.01 level while the strain x replication x time interaction was significant at the 0.001 level. The mean ethanol produced by each strain for each replication is presented in Table 7 to illustrate this. Examination of these means by ranking shows inversion of ordering between strains 1, 2 and 3 among the replications. This illustrates the fact that, while the variation among strains over replications was not significant, the manner in which the variation occurred was significant when measured against the variation incurred in the three-way interaction including time.

Of major importance in the analysis of variance of ethanol production in Table 6 is the fact that time was significant at the 0.001 level. This would be expected since the conversion of glucose to ethanol is not instantaneous, but is the result of the life processes and growth of the yeasts (30). Figure 6 illustrates mean ethanol production over time.

TABLE 6

ANALYSIS OF VARIANCE: PRODUCTION OF ETHANOL OVER TIME BY THREE YEASTS IN PUMPKIN MASH

Source	df	ms	f-ratio
Strain	2	0.2276	1.45^{ns}
Replication	2	0.0264	0.168^{ns}
S x R (error a)	4	0.15695	5.6**
Time	7	0.6373	22.82***
S x T	14	0.0355	1.27^{ns}
S x R x T (error b)	42	0.0279	16.24***
Residual	72	0.0017	

^{ns}Not significant at the 0.05 level.

**Significant at the 0.01 level.

***Significant at the 0.001 level.

TABLE 7

ETHANOL PRODUCED BY THREE YEAST STRAINS IN THREE FERMENTATIONS OF LADY GODIVA PUMPKIN FLESH

Strain	Replication[a]		
	1	2	3
	%		
1	0.94	0.78	0.89
2	0.64	0.81	0.74
3	0.90	0.74	0.76

[a]Means over time (n=16).

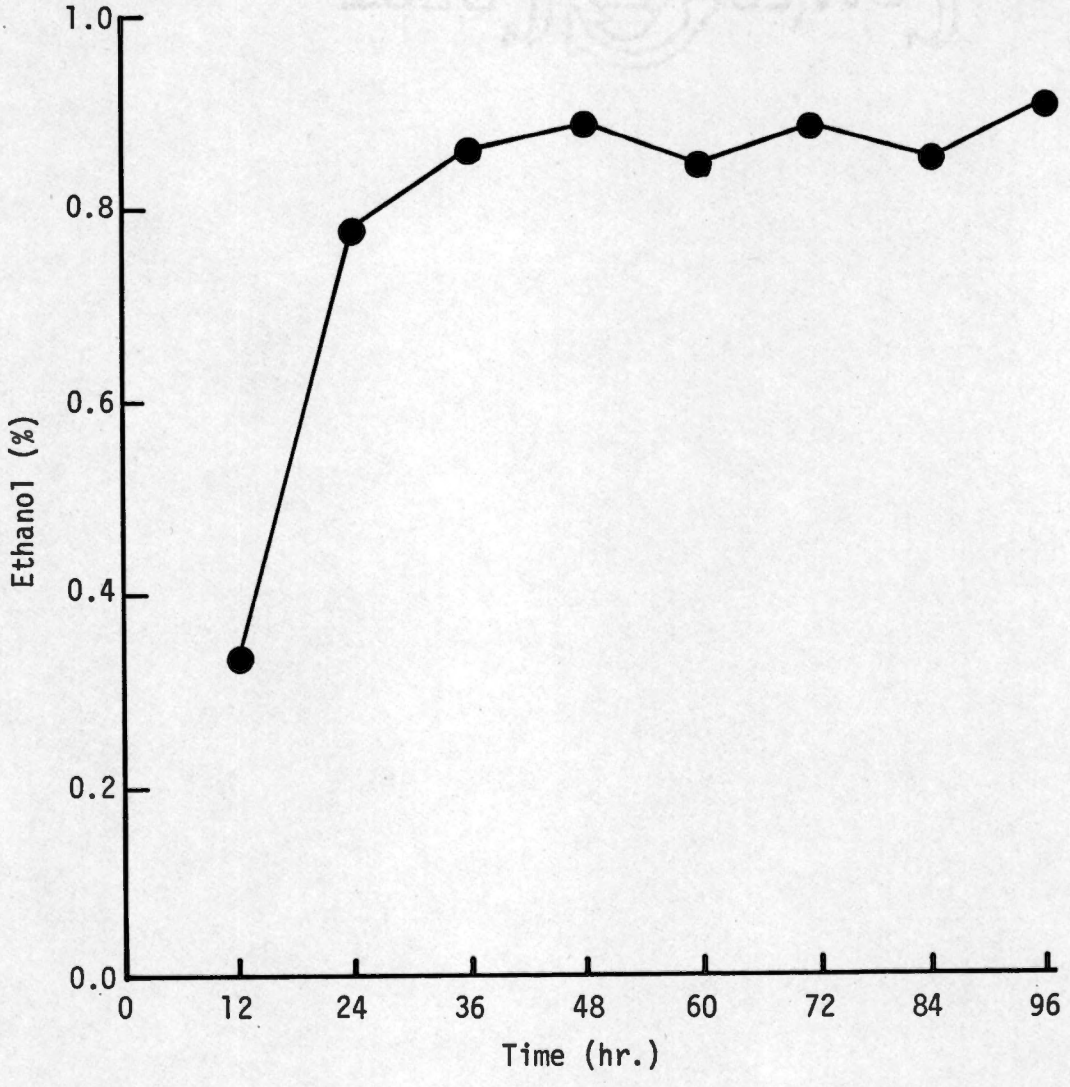

Figure 6. Ethanol Concentrations Over Time for Fermentations of Lady Godiva Pumpkin Flesh with Three Strains of Yeast.

Since time was a significant factor, the means over strain and replication were calculated and significant difference among these means was measured by Tukey's Test (39). Table 8 shows these means and differences at the 0.05 level. It can be seen that the mean ethanol concentration at 12 hours was significantly different, while means of 24 hours through 96 hours were not significantly different. These results suggest that fermentation of Lady Godiva pumpkin flesh can be considered completed after 24 hours under the conditions of this experiment. This is not unreasonable to expect when considering the relatively low concentration of sugars in the raw material, 5.81% NFE on a wet basis. It must also be kept in mind that this concentration was reduced by half by blending with water for fermentation. Enzymatic hydrolysis must also be considered. If the expected hydrolysis of cellulose to glucose occurred, this also should be completed within 24 hours since preliminary evaluations of Cellulase Tv Concentrate showed a maximum increase in soluble sugars at 24 hours. The mean of the time means which were not significantly different, 24 hours through 96 hours, was calculated and taken as an estimate of the true mean of ethanol production from fermentation of Lady Godiva pumpkin flesh. This value came out to be 0.86%. Although this value seems low, when considering the conditions of the fermentation and the possibility of such fermentations on a large scale, it seems that Lady Godiva pumpkin flesh can be considered a viable source of ethanol. This will be discussed in more depth when considering the results of the compositional analyses of the pumpkin mash before and after fermentation.

TABLE 8

ETHANOL PRODUCTION OVER TIME; THREE YEASTS, THREE REPLICATIONS

Time hours	Ethanol[a] %	
12	0.34	A
24	0.79	B
36	0.87	B
48	0.90	B
60	0.85	B
72	0.88	B
84	0.85	B
96	0.91	B

[a]Means include duplicate samples. Means followed by the same letter are not significantly different, $P < 0.05$ ($n=18$), by Tukey's Test (39).

IV. PROXIMATE ANALYSIS OF LADY GODIVA PUMPKIN FLESH MASH BEFORE AND AFTER FERMENTATIONS

A complete proximate analysis was done on each pumpkin flesh mash before and after each fermentation, as described in the Materials and Methods section. Each analysis was done in duplicate, except moisture which was done in single, large batches. Analysis of variance was carried out on the results for each component (moisture, protein, fat, fiber, ash and NFE) to determine areas of significant difference. In the discussion of these results, time is in reference to before and after fermentation, while replication is in reference to actual fermentation replication.

All concentrations were calculated on a dry matter basis except NFE which was calculated on a dry matter basis and a wet basis.

Table 9 shows the analysis of variance for moisture content. It can be seen that neither strain nor replication was significant at the 0.05 level. Time was significant at the 0.001 level. Mean moisture contents were 89.29% before fermentation and 94.13% after fermentation. This increase can be accounted for by the production of ethanol and other volatiles, which would be evaporated along with moisture. This increase was responsible for the difference in before and after samples. These results were expected. Since each strain produced similar amounts of ethanol, similar increases in moisture would also be expected.

Table 10 gives the analysis of variance with protein content in pumpkin flesh mash before and after each fermentation. This was calculated on duplicate determinations of three yeast strains, three replications and two times. It can be noted that strain, replication, strain x

TABLE 9

ANALYSIS OF VARIANCE OF MOISTURE CONTENT OF PUMPKIN FLESH MASH BEFORE AND AFTER FERMENTATION

Source	df	ms	f-ratio
Strain	2	0.1825	0.5798^{ns}
Replication	2	0.581	1.8459^{ns}
Time	1	108.15	343.61***
Error	12	0.31475	

^{ns}Not significant.

***Significant at the 0.001 level.

TABLE 10

ANALYSIS OF VARIANCE OF PROTEIN CONTENT OF PUMPKIN FLESH MASH BEFORE AND AFTER FERMENTATION[a]

Source	df	ms	f-ratio
Strain	2	1.9580	1.43[ns]
Replication	2	1.2563	0.92[ns]
S x R (error a)	4	1.3675	1.56[ns]
Time	1	554.9951	634.56***
S x T	2	0.2012	0.23[ns]
S x R x T (error b)	6	0.8746	0.3782[ns]
Residual	18	0.7653416	

[ns]Not significant at the 0.05 level.

***Significant at the 0.001 level.

[a]Compositional analyses for fat, ash, fiber and NFE were evaluated by the same ANOVA model.

replication interaction, strain x time interaction and the strain x replication x time interaction are not significantly different at the 0.05 level. Time was signifcant at the 0.001 level. Mean protein concentrations for time were 11.63% before fermentation and 19.49% after fermentation. This increase can be explained by the accumulation of yeast cells during fermentation, since yeast cells are largely protein.

The compositional analyses for fat, ash, fiber and nitrogen-free extract (NFE) were evaluated by the same analysis of variance model as used for protein. Therefore, these analyses are not presented in tables, but the results will be discussed individually.

In the analysis of variance for fat concentration, no main effects or interactions were found to be significantly different at the 0.05 level. Mean fat concentrations were 1.85% before fermentation and 1.91% after fermentation. No changes in lipid content were expected during fermentation and these results show that none occurred.

In the split plot analysis of variance for ash content, time was found to be significantly different at the 0.001 level. Other sources were not significantly different at the 0.05 level. Mean ash concentrations were 7.90% before fermentation and 14.04% after fermentation. The accumulation of carbonates from the CO_2 produced during fermentation in the acid (pH 5.0) medium would account for the increase in ash.

In analysis of variance of fiber (NDF) content of pumpkin flesh mashes before and after fermentation, no sources were found to be significantly different at the 0.05 level. Mean fiber content before fermentation was 23.20% and 22.38% after fermentation. These results were not as expected. It was hoped that through hydrolysis of cellulose, with the Cellulase Tv Concentrate enzyme, the fermentable sugars could be

increased and therefore the total fiber content reduced. Since no significant difference was found in the before and after samples, this suggests that the Cellulase Tv Concentrate had no effect on the cellulose. This could be explained by the difficulty of mixing the enzyme solution into the pumpkin flesh mash. Enzyme solutions were initially evaluated by placing flasks of the enzyme and pumpkin slurry mix in a shaker bath. This assured a complete and thorough mixing and saturation of the enzyme with substrate. When enzyme solutions were added to actual fermentation mashes, these could only be shaken by hand while in a closed flask. Due to the viscosity of the pumpkin and water slurry, this mixing likely did not distribute the enzyme throughout the suspension and did not saturate the enzyme with substrate. Although no significant difference was noted in the fiber content of pumpkin mash before and after fermentation, it is interesting to note that the mean value was less after fermentation.

The analysis of variance for NFE (dry matter basis) showed that the only significant source of variations was time (0.001 level). Mean NFE (dmb) concentrations were 55.41% before fermentation and 42.23% after fermentation. NFE means on a wet basis were 5.94% before fermentation and 2.48% after fermentation. These substantial decreases in NFE concentration were expected since carbohydrates are the carbon sources for ethanol production.

Table 11 gives the composition of Lady Godiva pumpkin flesh mash before and after fermentations. Any means significantly different at the 0.05 level are noted.

When evaluating the complete fermentation process, the amount of ethanol produced should be compared to the amount of carbohydrate lost.

TABLE 11

COMPOSITION OF PUMPKIN FLESH MASH BEFORE AND AFTER FERMENTATION

Component[1]	Concentration[2] Before	After
	%	
Moisture	82.29 a	94.13 b
Protein	11.86 a	19.42 b
Fat	1.85 a	1.91 a
Ash	7.90 a	14.04 b
Fiber	23.20 a	22.38 a
NFE (dry basis)	55.41 a	42.23 b
NFE (wet basis)	5.94 a	2.48 b

[1]Components except moisture and NFE (wet basis) reported on a dry matter basis.

[2]Means of 3 yeast strains, 3 reps, duplicate analyses (except moisture); means in rows followed by the same letter are not significantly different at $P < 0.05$.

By using the mean NFE (wet basis) concentrations of the pumpkin flesh mash before and after fermentation, a decrease in carbohydrate content of 3.46% is noted. Since Lady Godiva pumpkin flesh was blended 1:1 (w/w) with water, this was actually a decrease of 1.73% in the mash. That is to say during fermentation the original sugar concentration was diluted by half and thus, percentage-wise, 50% less carbohydrate was available for fermentation. It has been stated that during actual fermentation about 51.1% of the sugar present is theoretically convertible to ethanol with the remaining 48.9% going to carbon dioxide (35). Using the 1.73% decrease in NFE and the 51.1% theoretical yield, an ethanol content of 0.88% would be expected. The mean ethanol content calculated from the means over time (excluding 12 hours) was 0.86%. Using these two values, 97.73% of the theoretical yield was achieved during these fermentations.

Based on the achievement of a high percentage of the theoretical yield of ethanol and the fact that fermentation was completed after 24 hours, it appears that Lady Godiva pumpkin flesh represents a feasible source of ethanol. These experiments were carried out on a laboratory scale and further investigation would be necessary to evaluate this on a large scale. Ethanol produced could be recovered by a simple distillation process. This ethanol would then represent a good potential energy source, mainly for the addition to gasoline to form gasohol. This would be one of several potential uses for the ethanol produced, but there would still be a pumpkin mash left as a byproduct.

Due to the relatively high concentration of protein, 19.42%, the dry material left from this fermentation could be utilized as a possible

animal feed or animal feed supplement. If necessary the ethanol produced during fermentation could be recovered and used to help fuel a drying system for the mash.

CHAPTER V

SUMMARY

The objective of this investigation was to evaluate the potential of producing ethanol from the flesh of the Lady Godiva pumpkin (Cucurbita pepo L.) by yeast fermentation combined with enzymatic hydrolysis of the cellulose constituent to increase total sugars.

The proximate composition of the Lady Godiva pumpkin flesh was determined. The flesh was found to contain 90.36% moisture, and on a dry matter basis, 11.34% protein, 8.34% ash, 1.76% lipid, 18.67% fiber (neutral detergent fiber) and 59.89% nitrogen-free extract (NFE). NFE on a wet basis was 5.81%. Additional compositional analyses on dried Lady Godiva pumpkin flesh showed 12.77% acid detergent fiber, 0.96% acid detergent lignin, 11.80% cellulose, 5.90% hemicellulose, 42.63% total soluble sugars and 1.05% starch. Total soluble sugars was 4.11% on a wet basis.

Three separate enzyme complexes, Cellulase C, Rohalase M-4 and Cellulase Tv Concentrate, were evaluated for hydrolysis of the cellulose component into glucose. In a pumpkin flesh: water slurry, Cellulase C showed a mean increase in total sugars of 0.90%, Rohalase M-4 gave a 1.03% mean increase and Cellulase Tv Concentrate gave a 1.49% mean increase. Due to this larger mean increase in total sugars and the fact that the hydrolysis was completed after 24 hours at 30°C, Cellulase Tv Concentrate was chosen for use during fermentation of Lady Godiva pumpkin flesh.

Four yeast strains, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Saccharomyces cerevisiae var. gentry and Schizosaccharomyces octosporus, were evaluated for ethanol production in a 5% glucose broth. The three Saccharomyces strains were chosen for use in fermentation of Lady Godiva pumpkin flesh due to their higher ethanol production abilities.

Fermentations were carried out using a pumpkin flesh in water suspension (1:1 w/w) with 24-hour cultures of yeast added at a 1.0% level. Ethanol production was measured by gas solid chromatography in samples taken at 12-hour intervals over 96 hours. Neither strain nor replication were found to be significantly different (0.05 level). Time (0.001 level), strain x replication interaction (0.01 level) and the strain x time interaction (0.001 level) were all found to be significant. For overall time means of ethanol production only the 12-hour mean was found to be significantly different (0.05 level). The mean ethanol concentration produced, using the not significantly different means, was 0.86%.

Proximate composition of each pumpkin flesh mash before and after fermentations was determined. Moisture content was 89.29% before and 94.13% after fermentation, and on a dry matter basis, protein content was 11.86% before and 19.42% after fermentation, percentage fat was 1.85% before and 1.91% after, ash content was 7.90% before and 14.04% after fermentation, fiber content was 23.20% before and 22.38% after fermentation and nitrogen-free extract (NFE) was 55.41% before and 42.23% after fermentation. NFE, on a wet basis, decreased from 5.94% before fermentation to 2.48% after fermentation. Of these means

moisture, protein and ash showed significant (0.05 level) increases after fermentation while NFE (wet and dry basis) showed a significant (0.05 level) decrease after fermentation.

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