Reduction of steeping time in wet corn milling by enzyme treatment

by

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INTRODUCTION

The corn wet milling industry began in the United States in 1844. Colgate and Company built two small starch factories at Jersey City, New Jersey and Columbus, Ohio. Thomas Kingsford was the plant supervisor at the Jersey City plant before leaving to start the much larger Kingsford Cornstarch Plant in Oswego, New York in 1848. Kingsford has been labeled as the father of the corn wet milling.

By the early 1900s, formation of most of the present wet milling companies had occurred. Today there are eleven major corn wet milling companies in the United States.

The wet milling process remained rather archaic for 125 years until the late 1960s when the Japanese developed new enzyme technology to economically convert glucose to fructose. As a result of this invention, demand increased for starch and need for technological improvements in wet milling machinery increased. The 1970s became what has been labeled by wet millers as "The Great Modernization". During this period, germ flotation tanks were replaced with banks of hydroclones; nylon screen shakers, with Dutch State Mine (DSM) screens; and starch tables, with Merco centrifuges and hydroclones.

From its origins as a small, labor-intensive process primarily concerned with only producing relatively pure starch, corn wet milling developed into an industry that seeks optimum

use and maximum value from each constituent of the corn kernel. Today, products produced by the wet milling of a 56-pound bushel of corn provides (Iowa Corn Promotion Board, 1988):

* enough high-fructose corn syrup to sweeten 325 cans of soft drinks;
* enough corn oil to make two pounds of margarine;
* more than enough starch to produce a ton of paper;
* 15 pounds of carbon dioxide to put the "fizz" in carbonated beverages;
* 2.5 gallons of fuel alcohol for blending with gasoline; and
* enough protein meal to raise three chickens.

This is just a small sample of the thousands of uses for refined corn constituents. The push is on to explore new uses for these constituents. Hopefully, as a result of these explorations, will come an increase in demand for more corn which will play an integral part in rebuilding and strengthening the agricultural economy. More cost-effective milling technologies will play a critical role in further expansion of corn utilization.

Major advances in equipment design and performance have already been made and it is not likely additional major advances are possible. New approaches to wet milling are required to improve cost-effectiveness. One approach to improving the wet milling process is through employing advances in bioprocessing to reduce capitol and labor costs. This study

focuses on the reduction of the steeping requirement (the second greatest expense after purchasing corn) and enhancement of starch release by the use of enzymes. One objective is to develop a laboratory procedure which closely simulates commercial practice, especially the steeping step. Another objective is to evaluate the feasibility of incorporating enzymes into the steep solution to reduce the steep time and enhance starch/gluten separation without materially altering the process currently in commercial practice.

LITERATURE REVIEW

Corn Wet Milling

The basic constituents of the corn kernel

The amounts of individual constituents of corn kernels vary from harvest to harvest, from one variety to another, from one growing location to another, and by the amount of moisture retained by the kernel. No. 2 yellow dent corn is normally purchased by the corn refining industry in the United States (USDA, 1970). Dent corn like other types of corn is composed of four main parts; the tip cap, the fibrous outer layer or pericarp (also commonly called the hull or bran), the endosperm, and the germ (Figure 1).

The germ makes up 10 - 12% of the dry kernel weight (Table 1). Most of the oil, and much of the protein, solubles, sugars, and ash are contained in the germ.

The main energy reserve of the kernel is the endosperm. It comprises 82 - 89% of the dry kernel weight and is 86 - 89% starch by weight. The endosperm consists of starch granules embedded in a continuous matrix of protein surrounding each granule (Wolf et al., 1952a; Wolf and Khoo, 1975). There are two types of endosperm, floury and horny. The floury endosperm is easily separated from the kernel and is opaque due to rupturing of the thin strands of protein matrix upon drying

Figure 1. Structure and cross section of the corn kernel. (Modified from Hoseney, 1986)

BRAN

ENDOSPERM

GERM

TIP CAP

BRAN
Epidermis
Mesocarp
Cross Cells
Tube Cells
Seed Coat (Testa)
Aleurone Layer (part of endosperm but separated with bran)

ENDOSPERM
Horny Endosperm
Floury Endosperm
Cells Filled with Starch Granules in Protein Matrix
Walls of Cells

GERM
Scutellum
Plumule or Rudimentary Shoot and Leaves
Radicle or Primary Root

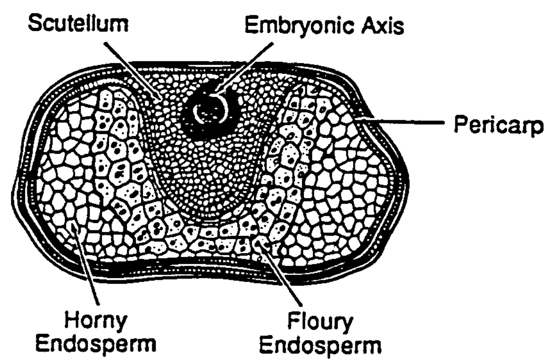


Table 1. Weight and Composition of component parts of dent kernels from seven midwest hybrids (Earle et al, 1946)

Part	% dry wt. of whole kernel	Composition of kernel parts (% d.b.)			
		Starch	Fat	Protein	Ash & sugar
Endosperm					
Mean	82.9	87.6	0.80	8.0	0.92
Range	81.8-83.5	86.4-88.9	0.7-1.0	6.9-10.4	0.7-1.3
Germ					
Mean	11.1	8.3	33.2	18.4	21.3
Range	10.2-11.9	5.1-10.0	31.1-35.1	17.3-19.0	19.9-23.8
Pericarp					
Mean	5.3	7.3	1.0	3.7	1.14
Range	5.1-5.7	3.5-10.4	0.7-1.2	2.9-3.9	0.6-1.4
Tip cap					
Mean	0.8	5.3	3.8	9.1	3.2
Range	0.8-1.1	...	3.7-3.9	9.1-10.7	1.4-2.0
Whole kernel					
Mean	100.0	73.4	4.4	9.1	3.3
Range	...	67.8-74.0	3.9-5.8	1.37-1.5	1.61-2.22

(Duvick, 1961). In the horny endosperm, the thicker protein matrix shrinks during drying but does not rupture. The resulting pressure forms a dense glassy structure.

The outermost covering of the kernel is composed of multiple layers collectively termed the pericarp. The pericarp makes up 5 - 6% of the dry kernel weight and extends to the base of the kernel protecting the seed. At the base it unites

with the tip cap. The tip cap is a remnant of the seeds attachment to the cob. It constitutes 1% of the dry kernel weight.

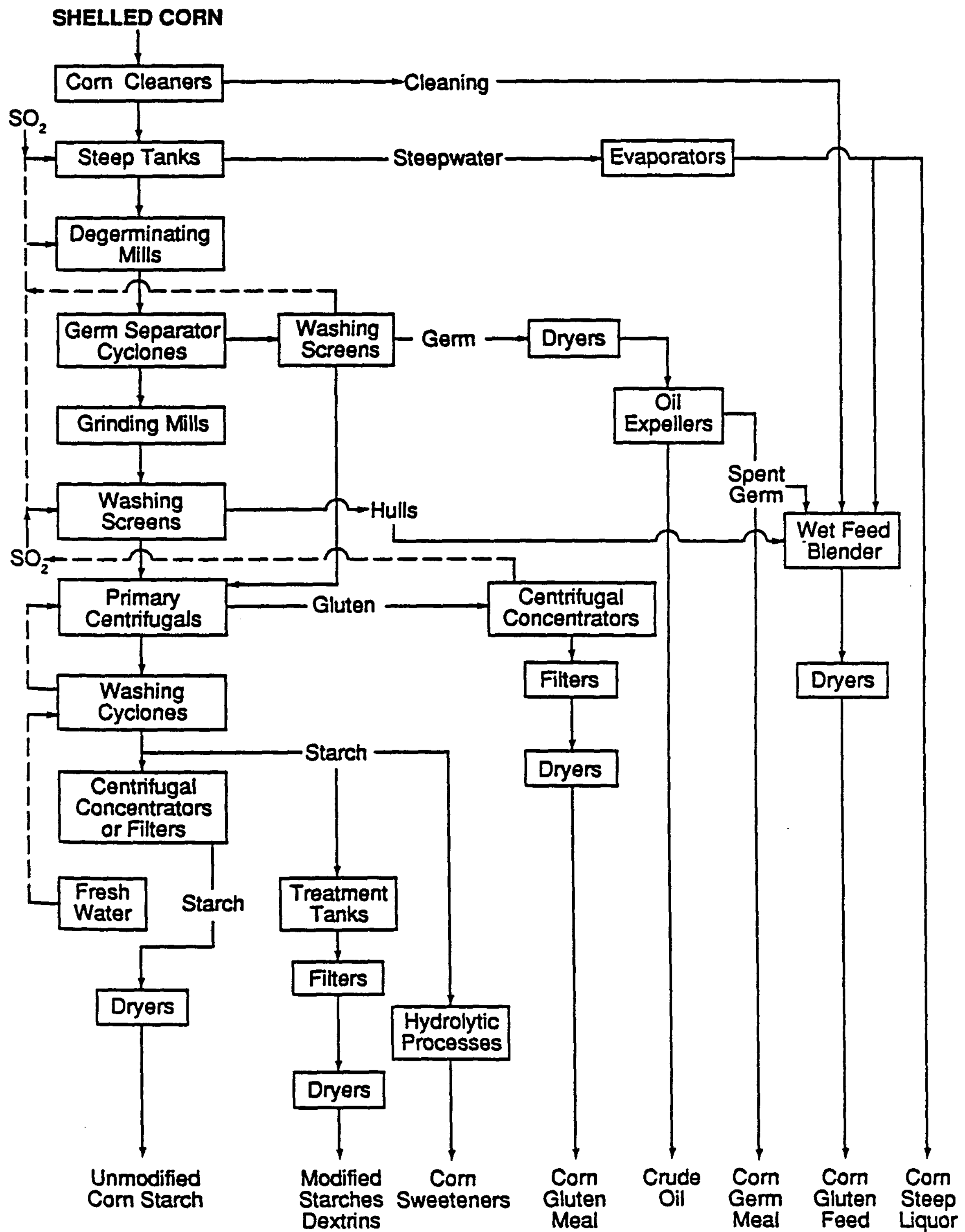
The industrial process

The objective of the corn wet milling process is to separate corn kernels into component parts, to achieve maximum purity for each constituent, and to complete the process with a minimum of energy usage.

The milling process (Figure 2) has been best characterized by Watson (1984) and divided into two major steps. The first phase involves chemical changes to the kernel while the second phase involves a sequence of mechanical separations.

Steeping, soaking in water, is probably the first and most important step in the wet milling process; without a proper steep, quality of the end products will suffer greatly. This is the first phase of the milling process and results in chemical transformations of some of the corn constituents. Corn is placed into large tanks holding 2000 - 13,000 bushels each. Chemical changes are brought about by soaking the corn in steep water for 36 - 50 hrs at 48° - 52°C. In industry, steeping is not a batch process but rather a countercurrent semi-continuous system of six to twelve tanks (steeps). Countercurrent steeping involves adding downstream process water containing 0.1 - 0.2% sulfur dioxide (SO_2) to corn that

Figure 2. Flow diagram of the corn wet milling process. (Modified from Watson, 1984)


SHELLED CORN
Corn Cleaners
Cleaning
SO_2
Steep Tanks
Steepwater
Evaporators
Degerminating Mills
Germ Separator Cyclones
Washing Screens
Germ
Dryers
Oil Expellers
Grinding Mills
Spent Germ
Washing Screens
Hulls
Wet Feed Blender
SO_2
Primary Centrifugals
Gluten
Centrifugal Concentrators
Filters
Dryers
Washing Cyclones
Dryers
Starch
Centrifugal Concentrators or Filters
Fresh Water
Starch
Treatment Tanks
Filters
Hydrolytic Processes
Dryers
Dryers
Unmodified Corn Starch
Modified Starches Dextrins
Corn Sweeteners
Corn Gluten Meal
Crude Oil
Corn Germ Meal
Corn Gluten Feed
Corn Steep Liquor

has been in the system the longest, usually 40 hrs into the steep. Each subsequent tank receives steepwater from the previous steep until the steepwater finally emerges from the bottom of the steep tank containing corn that has been soaked for the least amount of time. At this point, the water is withdrawn from the system as light steepwater (low in SO_2 content) and concentrated to 40 - 50% solids for utilization as a fermentation media or as an ingredient in corn gluten feed.

Approximately 30 - 34 L (8 - 9 gal) of water is used per bushel of corn during the steeping process (May, 1987). Of this total, 13 L (3.5 gal) of steepwater are absorbed per bushel of corn increasing the moisture from about 15.5 to 45%. The remaining 17 - 21 L (4.5 - 5.5 gal) are removed from the system as steep liquor. About 1.3 - 1.6 kg (2.8 - 3.6 lbs) of solids per bushel are extracted from the corn by the steepwater.

The second step of the wet milling process involves the mechanical action of grinding and separating. Separation is accomplished by differences in particle sizes and densities of component parts. Steeped corn is sluiced into attrition mills to free the germ and macerate the endosperm. Because the germ contains 40 - 50% oil, it has a lower density than the remainder of the kernel and is separated with a liquid cyclone separator (hydroclone). The isolated germ is washed and dried in preparation for oil extraction.

After germ separation, the remaining slurry is sieved. The larger particles not passing through the screen, the hulls and chunks of horny endosperm, are reground with an impact mill. This ground portion travels through a second series of screens (DSM screens) designed to remove as much fiber from the slurry of starch and protein as possible. Fiber that is removed by the screen is washed, dewatered, and dried for use in corn gluten feed.

The material passing through the DSM screen, known as "mill starch", is pumped into a bowl-type centrifuge for primary separation of starch and gluten. The less dense gluten is dewatered and dried. In this form it is known as corn gluten meal.

Mill starch still contains 1 - 2% protein after primary separation. It is then subjected to a second separation using a large number of very small hydroclones which further reduce the level of protein. The purified starch is utilized in slurry form or dewatered and dried to produce common, unmodified corn starch.

The products

Laboratory steeping and milling procedures give results close to those obtained in commercial wet milling and in some fractions give superior results when considering the separating equipment available to industrial wet millers today. Superior

purity of the germ and fiber fractions have been achieved in the laboratory because of more complete removal of the germ from the corn slurry and more efficient rinsing of the fiber in the fiber separation step. However, industrial hydroclones provide superior starch and protein separation which increases starch yield, produces gluten of higher purity, and eliminates the squeegee fraction (a small fraction of difficult to separate starch/gluten). Typical yield and composition of industrial wet milled products are given in Table 2.

The Biochemistry of Corn Steeping

Water absorption and release of solubles

The mechanism of water absorption has been characterized by both Cox *et al*. (1944) and Wolf *et al*. (1952b). They have shown that liquid enters the kernel through the tip cap and quickly moves upward through voids in the pericarp. The water reaches the dent crown region at the top of the kernel before any liquid begins to penetrate through the aleurone layer to the starchy endosperm at the sides of the kernel. Simultaneously, the germ is penetrated and wetting is complete in 4 hrs at 49°C. After 8 hrs, the steeping liquid has completely diffused throughout the endosperm. Although the mass movement of water into the kernel is relatively rapid, the kernel is not completely softened at this time. Hydration of

Table 2. Typical yield and composition of industrial and laboratory wet milled products (from Anderson, 1963 and Anderson and Watson, 1982)

Product	Industrial(% d.b.)	Laboratory(% d.b.)
Germ		
Fraction yield	7.5	6.2
Starch	10.0	30.6
Protein	12.0	23.7
Fat	52.0	NA[a]
Oil yield	3.9	4.5
Fiber		
Fraction yield	11.5	12.5
Starch	23.0	19.7
Protein	12.0	14.7
Fat	1.0	NA
Starch		
Fraction yield	67.5	65.4
Starch	99.0	NA
Protein	0.30	0.54
Fat	0.02	NA
Crude fiber	0.03	NA
Gluten		
Fraction yield	5.8	8.1
Starch	14.6	26.6
Protein	65.8	42.9
Fat	6.2	NA
Squeegee[b]		
Fraction yield	...	1.4
Protein	...	20.8
Solubles		
Fraction yield	7.5	7.1
Protein	46.0	33.7

[a]Denotes data not available.

[b]Equivalent to inseparables fraction. This fraction is not produced in industry.

cellular components required for softening begins in the range of 12 - 18 hrs (Watson, 1984).

Watson et al. (1955a) and Franzke and Wahl (1970) found that the extraction of dry solids begins and is most rapid during the cellular hydration period. This is believed to be due to the absorption of lactic acid in combination with the high temperature of the steep which causes the death of the cells in the germ and an increase in the porosity of the membranes. About one-half of the steepwater solubles results from the germ, even though this constituent comprises only 11 - 12% of the total kernel weight (Table 1). This is verified by the fact that the oil content of the raw germ increases from 30 - 38% to 50 - 60% after steeping (Table 2). A large assortment of molecules, such as sugars, amino acids, protein, and minerals, are leached into the steepwater. The efficiency of the steep and the sequence of events during the process can be determined by monitoring these solubles.

The action of sulfur dioxide

The majority of the remaining steepwater solubles are leached from the endosperm after the action of SO_2. The solubles liberated as a consequence of the SO_2 are noticeable at approximately 12 hrs into the steep and continue up until the time the corn is removed from the steep. However, this occurs at a much slower rate than those released from the germ.

Originally, SO_2 was added as an inhibitor to putrefactive organisms, but SO_2 was later recognized to play an active chemical role in the steeping process.

It was 100 years after the first corn wet milling plant went into operation that Cox and co-workers (1944) first recognized the reducing effects of SO_2 on the protein matrix of the endosperm. This is now believed to be the most important effect of SO_2 addition. Over a period of 24 hrs at 50°C, they demonstrated that the protein matrix gradually swells, forms globules, and disperses. They discounted the acidic nature of SO_2 as a contributing factor in the disintegration of protein by substituting lactic, acetic, and hydrochloric acid in equivalent concentration, or by acetic or hydrochloric acid at the same pH. It was discovered that addition of lactic acid to a steep containing SO_2 uniquely increased softening of the kernel.

What was observed under light microscopy was later clarified by scanning electron microscopy (SEM). Earp _et al_. (1985) conducted a study in which samples of steeped corn were withdrawn from commercial steeps at 0, 16, 20, 22, 24, 26, 28, 30, and 48 hrs and prepared for SEM. They observed structural changes in the protein matrix (holes) and in the cell walls (depressions rather than holes) after 18 - 20 hrs. Although the protein matrix underwent some degradation after 20 to 30 hrs of steeping, much of the matrix remained intact. It was

not until 48 hrs that the matrix was totally dispersed.

Christianson et al. (1969) and Wall and Paulis (1978) characterized the protein matrix surrounding the starch granules as primarily a large and complex glutelin protein containing many inter- and intramolecular disulfide bonds. These disulfide bonds were reduced to free sulfhydryls by the sulfite or bisulfite ion formed in water by SO_2.

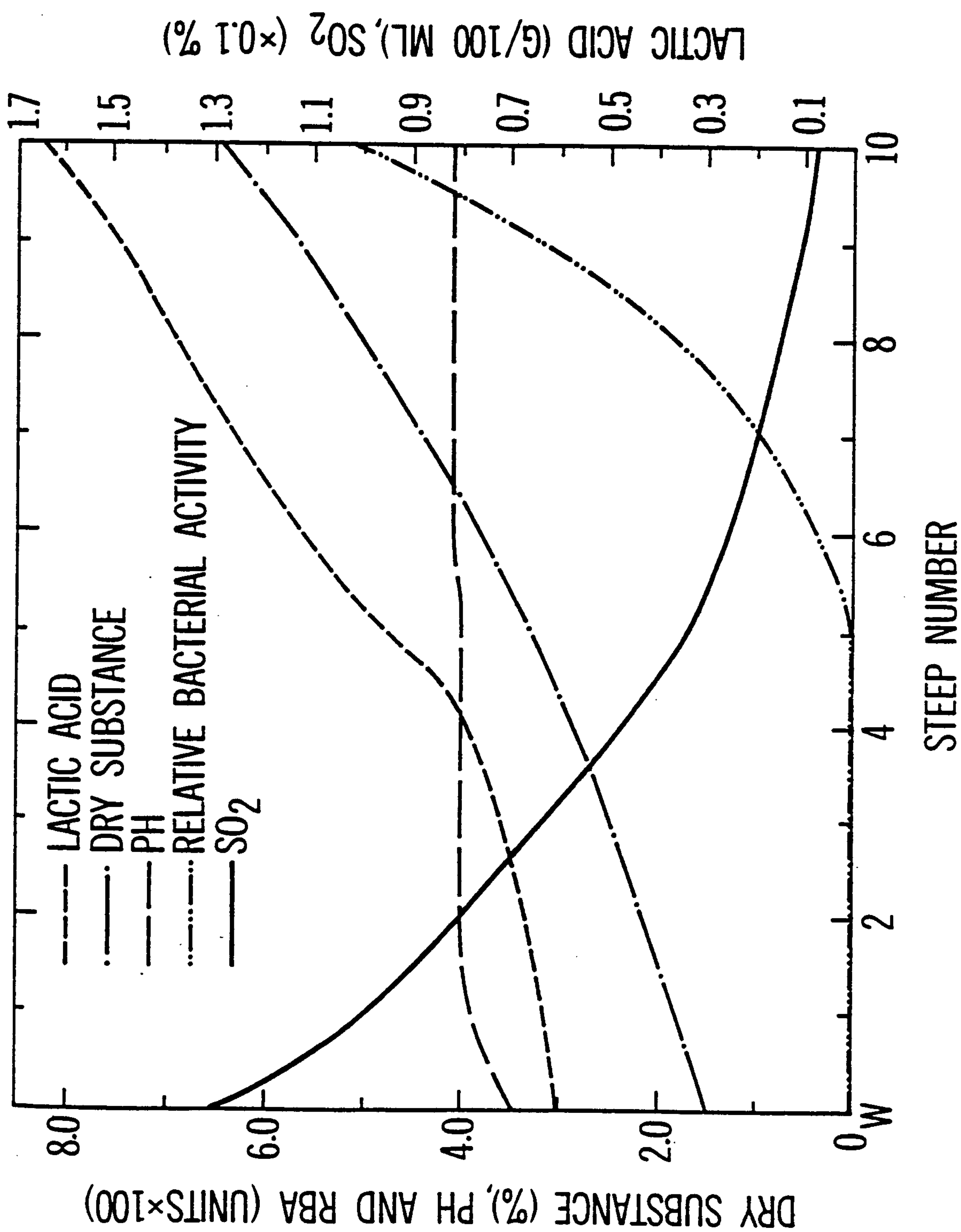
SO_2 was also found to increase the rate of water diffusion into the corn kernel. During the first 8 hrs of steeping at 49°C, Fan et al. (1965) determined that corn absorbed 20% more water in SO_2 solution than without.

The effect of the lactic acid bacteria

The final aspect of the biochemistry of corn steeping deals with the role of homofermentative lactic acid bacteria. Many investigators have studied these bacteria. Watson and co-workers (1955a) examined the conditions necessary for proliferation of lactic acid producing species. These conditions were 45° - 55°C with a pH near 5. They also monitored the progress of the steeping process using the relative bacterial activity (RBA) to relate the amount of lactic acid formed to the population of lactic acid bacteria. Figure 3 is a plot of steepwater composition illustrating the events that occur across a ten-steep, continuous-flow battery.

The acid these bacteria produce is believed to be

Figure 3. Composition of steepwater in individual steeps of a continuous-flow countercurrent steep battery. "W" under steep number is composition of steep input water. Water flow is from left to right. Steep number 10 contains newest corn. (Watson, 1984)



LACTIC ACID
DRY SUBSTANCE
PH
RELATIVE BACTERIAL ACTIVITY
SO2
STEEP NUMBER
DRY SUBSTANCE (%), PH AND RBA (UNITS×100)
LACTIC ACID (G/100 ML), SO2 (×0.1 %)
8.0
6.0
4.0
2.0
0
W
2
4
6
8
10
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1

important in improving separation performance in the wet milling process by buffering of the steepwater between pH 3.8 and 4.1. In this pH range, undesirable microorganisms are inhibited. Lactobacilli, however, do not function well at this pH either, so it is not surprising that Wahl (1969) found that 70% of the bacterial activity occurs inside the kernel at the new grain end of the steeping battery where the pH is close to 5. Watson (1984) suggested that microorganisms occupy the spaces inside the tip cap where the sugar concentration is highest.

Lactic acid has been linked to softening of the kernel and protein matrix (Cox et al., 1944; Kerr 1950; Watson et al., 1955a), protein solubilization and dispersion (Roushdi et al., 1981a), and facilitating in the separation of the hull, fiber, and protein from steeped grains (Roushdi et al., 1981b). These effects may be explained by an observation made by Watson et al. (1955a) who found that 85% of the nitrogen in steepwater is dialyzable nonprotein nitrogen (NPN). Since one-tenth of the nitrogen in corn is in the form of NPN, it can account for only about one-fourth of the total soluble nitrogen. Therefore, they suggested that proteolytic enzymes of bacterial origin must be present. Lactobacilli are normally thought to be incapable of hydrolyzing protein. It may be that one or more of the non-lactobacilli are responsible or that a particular strain of lactobacilli has developed proteolytic enzymes.

Previous Investigations of Steeping

Methods of steeping

Steeping in the laboratory has been simulated either batchwise or countercurrently following methods described by Cox et al. (1944), Zipf et al. (1950), and Watson et al. (1951, 1955a, 1955b).

Many researchers have chosen batchwise steeping for their studies because it is convenient, reproducible, inexpensive, easy to monitor the biochemical factors occurring within the steep, and requires fewer man-hours for operation than a countercurrent system. This type of steeping has been performed in two ways. The first method involves soaking the corn without circulating the steeping medium while the second involves either continuous or discontinuous circulation. Published batch steeping methods are listed in Table 3.

Few attempts have been made to simulate countercurrent steeping in the laboratory. The reasons for this are: more expensive equipment is required; more man-hours for achieving steady-state operation are required; and difficulty in simulating the conditions that occur in an industrial system at the laboratory scale.

Watson et al. (1951) were the first investigators to describe a laboratory countercurrent system. Their steeping system consisted of 12 fruit jars holding 400 g of grain each

Table 3. Compilation of published batch steeping methods

Researcher	Steeping medium[a]	Circulation	Steeping Time (hr)	Sample size (g)/ solution amt. (ml)
Cox *et al.*, (1944)	0.1 - 0.4% SO_2 (gas)	continuous	24	400/1700
Zipf *et al.*, (1950)	0.2 - 0.25% SO_2 (gas)	continuous	24	1816/2800
Watson *et al.*, (1955a)	0.1 - 0.15% SO_2 ($Na_2O_5S_2$)	none	24 - 64	600/950
Watson *et al.*, (1955b)				
Step 1	0.05% SO_2 ($K_2O_5S_2$) + 1.5% lactic acid	discontinuous	40	350/1500
Step 2	0.1% SO_2 ($K_2O_5S_2$) + 0.5% lactic acid	continuous	8	
Anderson (1963)	0.2% SO_2 (gas)	continuous	48	1500/2800
Krochta *et al.*, (1981)	0.2% SO_2 ($NaHSO_3$)	continuous	48	300/600

[a]Source of SO_2 given in parentheses.

and fitted with a rubber stopper containing inlet and outlet tubes. The jars were kept at 50°C in a water bath.

The steeping medium entered this system from a supply

bottle and was delivered to the bottom of the first steep. The steepwater then moved up through the grain, overflowed into the bottom of the next steep, and proceeded on through the battery. Steepwater advanced through the system by application of vacuum at the end of the battery. The system was automated with a time clock which actuated a solenoid valve that opened the vacuum line. As a result, steepwater was drawn onto a new steep of dry grain and into the draw flask. The draw stopped when the electrodes in the top of the draw flask were contacted by the rising steepwater. Time clocks were set for intervals of 2 to 24 hrs which carried out as many as ten consecutive advances and draws by attaching a relay. The relay moved to a new position after each draw, disconnecting power to the preceding pair of electrodes and closing the circuit to the next set of electrodes in preparation for the next draw. After closer examination, this method appears to be more of a "batch advance" system later described by Watson (1984) than a true countercurrent system.

Neryng and Reilly (1984) describe a system that more closely simulates an industrial scale system, although details of its mechanics are lacking. They employed three 7-L baffled New Brunswick fermentors filled with 2.5 kg samples of corn. Each vessel as it came on line was charged with 3 L of steepwater and operated batchwise for 6 hrs. The supernatant during this time was stirred at 10 - 15 rpm. After 6 hrs,

fresh SO_2 solution was pumped into the top and removed from the bottom of the steep (this solution was not recirculated) for another 6 hrs. Steepwater drawn from the bottom of the previous steep was then pumped up to the top of the vessel for the remaining 12 hrs. The solutions flowed through the system at a rate of 160 ml/hr.

Methods of wet milling

All published laboratory wet milling methods can be traced to two sources, either Zipf et al. (1950) or Watson et al. (1951). The methods are very similar.

Both begin with degermination of the corn. Watson used a Waring Blendor to free the germ from the endosperm while Zipf used a Quaker City drug mill. The floating germs were skimmed off with a wire screen and washed. The washings were returned to the process.

The next operation after degermination involved sieving the remaining slurry in order to separate the coarse material from the bulk of the starch-protein suspension. The coarse fraction was reground through the Quaker City mill to free starch and protein from the fiber. Discharge from the mill was once again sieved and washed with several liters of water. The material retained on the screen was collected as the fiber fraction.

The screened slurry was combined with the washings and

passed through a very fine mesh screen to isolate the fine fiber fraction. The fine fiber was washed as above.

The mill starch slurry was allowed to settle for several hours. Excess water was then decanted until the desired specific gravity was obtained. The settled gluten and starch were reslurried and passed slowly down a pitched channel iron "table". The gluten flowed over the settled starch and was washed from the table. In between the gluten and starch layer was an off-white layer composed mostly of starch with a small amount of attached protein matrix known as the "squeegee" or "inseparables" fraction that was rinsed from the table. The remaining starch was slurried and collected.

Attempts at improving the steeping process

The commercial steeping process in use today is not much different from that used many years ago. Although this process is effective for subsequent separation of starch and gluten, it suffers in terms of being time, capital, and energy intensive. In order to reduce processing costs, steeping time needs to be shortened. Shortening the time in the existing process, however, creates serious quality, physical, and mechanical problems.

Many attempts have been made to reduce steeping time and increase starch yield and purity. Some researchers have approached the challenge from a mechanical viewpoint while

others approached the problem with chemical and biochemical alterations.

Hassanean and Abdel-Wahed (1986) found that by stirring the grain within a countercurrent steep with large agitating blades they were able to reduce the steeping period to 10 hrs. Starch yield was increased by 1% and the protein content in starch was decreased from 1.0 to 0.77%. Adoption of this method by the industry, however, would required costly modifications to the steeping tanks in order to facilitate the movement of corn in a steep and would increase water and energy usage.

In a process patented by Gillenwater _et al_. (1971), the time required for steeping was reduced to 2 - 16 hrs by utilizing cracked corn kernels. No data concerning yields or protein content were given, but high losses of starch with the steep liquor would be expected.

Roushdi _et al_. (1979) studied the effects of scratching the pericarp of corn kernels on reducing the steeping time requirement. They found that the steeping period could be reduced 40% by scratching grains that were presteeped for 10 hrs. However, scratching kernels midway through the steeping operation is not practical.

In another experiment designed to reduce mass transfer barriers between the endosperm and steep solution, Krochta _et al_. (1981) used degerminated corn in an attempt to increase

starch yield. The yield based upon degermed corn increased by 3 - 4%, but this increase would be expected to be more than off-set by the starch lost during germ removal. No mention of the loss of starch with germs was made.

Even though the previous three modifications were found to allow shortened steeping periods, destroying the integrity of the kernel would increase the amount of starch and soluble materials released into the steep solution. This would require installation of a system for recovery of starch and insoluble protein. Also, excessive solubles in steepwater is known to foul evaporators.

Meuser _et al_. (1985) were able to increase starch yield and decrease the protein content of starch by steeping grains under pressure (218 psi) at 50°C without SO_2. The pressure allowed the corn to reach saturation in only 2 hrs compared to 8 - 12 hrs for corn steeped under atmospheric pressure. The use of a high-pressure disintegration valve after germ separation made this shortened steeping process possible. This process has been attempted at the industrial level in Europe but has not been widely accepted. Again, this design involves a large capital investment for retrofitting and increases energy usage.

Ensiling of corn prior to steeping was found to be advantageous by Neryng and Reilly (1984). The starch yield was found to be increased by almost 10% while the amount of protein

in the starch decreased by 24%. This superior steeping action was attributed to the fermentation that occurs in ensiled corn which resulted in increased lactic acid levels in the steeps.

Krochta _et al_. (1981) also studied two chemical modifications of steeping conditions to reduce energy consumption. The first experiment involved reducing the steep volume while maintaining the same absolute concentration of SO_2. The results gave starch yields comparable to corn steeped under normal conditions. Decreasing the steeping volume, however, may cause difficulty in germ separation and downstream processing water shortages. The second modification involved doubling the concentration of SO_2 which yielded 6% more starch. This adjustment would also have deleterious effects such as upsetting the delicate balance of lactic acid fermentation, creating more pollution problems, and increasing health risks to consumers of the co-products.

In a report by Grindel (1965), a different approach to steeping in regards to equipment design was tried. Corn grains pretreated in sulphuric acid moved through a pipe containing warm water and sodium bisulfite in 6 hr. Starch of suitable quality was produced.

Based upon evidence that proteolytic enzymes may play a role in disintegrating the matrix proteins during steeping, Roushdi _et al_. (1981c) studied the effects of two proteases on intact, scratched, and broken corn grains. The steeping period

was decreased by 50% in the case of broken grains while no effect was noticed on the scratched or intact kernels. The enzyme treatment also improved starch recovery and starch quality by decreasing its protein content. This study, however, was not performed under normal steeping pH (6 and 8 vs 4) and temperature (37°C vs 50°C). Even though positive effects were only observed with broken kernels (difficulties with using nonintact kernels were described earlier), the addition of enzymes to the steep containing intact grain warrants further investigation.

Previous Investigations of Enzyme Usage

One goal of the present study is to evaluate the feasibility of adding a mixture of enzymes into the steep to reduce steeping time and enhance starch and gluten separation. Addition of a single class of enzymes has been attempted such as the addition of proteases discussed previously, but never has an investigation utilized more than one class of enzymes at a time.

It is known that enzymes capable of hydrolyzing various membranes and matrices exist inside a corn kernel. Wahl and Barby (1971) determined that indigenous proteolytic enzymes reach maximum efficiency under steeping conditions. Wall and Paulis (1978) attributed some of the poor separation between

starch and protein of artificially dried heat damaged corn to the inactivation of these proteases. Van Twisk and Tegge (1968) reported that addition of papain, bromelain, and trypsin to degerminated corn resulted in glucose syrup of high purity suitable for medical purposes.

Vojnovich _et_ _al_. (1960) subjected starch slurries to 0.2 - 0.5% solution of pepsin and papain for 2 hrs and reduced protein contents in starch from 1.27 to 0.42%. Papain would be more appropriate for steeping conditions than pepsin because its maximum activity range (pH 4.0 to 6.0) is close to the pH range encountered in a steeping system (pH 3.8 to 4.1). Digestion of subcellular protein structures in thin sections of endosperm with pronase was accomplished by Wolf and Khoo (1975). Under these conditions the matrix protein was digested in 30 to 60 minutes.

Spanheimer _et_ _al_. (1972) found that a variety of proteolytic enzymes resulted in increased protein solubilization of corn grits. Plant and fungal proteases provided the best results. They also noted that a combination of bromelain and SO_2 gave greater performance than either treatment alone at the same pH. This was believed to be a synergistic effect. Bromelain, which is most active in the range of pH 3.7 to 5.5, would provide the greatest activity in the pH conditions encountered in a steeping vessel.

In the above investigations the protease treatments were

applied to exposed surfaces within the kernel, to starch, or to a dry-processed corn fraction. Only Roushdi _et_ _al_. (1981c) attempted to enhance separation with addition of proteolytic enzymes to a steep containing whole kernels, which proved to be unsuccessful. Therefore, a path to the endosperm must be provided.

Obstacles encountered along this path include the pericarp, cell membranes, and cell walls which are primarily composed of hemicellulose, cellulose, pectins, and other ß-glucans. The carbohydrases are capable of hydrolyzing the polysaccharides in these structures. Other important functions anticipated from these carbohydrases include softening effects to the kernel and releasing bound and trapped starch. In promotional literature[1], Genencor, Inc. has claimed that after the addition of cellulase to the first grind mill in a commercial corn wet milling plant, bound starch decreased from 18.4 to 8.75%. They also claimed an increase in starch yield, an increase in grind capacity, and a decrease in steeping time with no detrimental effects on starch separation efficiency.

Sosulski _et_ _al_. (1988) also provided some encouraging results from an investigation concerning enhancement of oil extracted from canola seed by carbohydrase addition. They

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[1]Promotional literature concerning the evaluation of Genencor Cytolase™ 123 in corn wet milling available from Genencor, Inc., South San Francisco, CA.

found that these cell wall and membrane degrading enzymes (ß-glucanase, pectinase, hemicellulase, and cellulase) increased oil extraction rate and increased oil yield.

Objectives of This Study

The research objectives of this study are: 1) to develop a laboratory corn wet milling procedure which closely simulates that in commercial practice, especially the steeping phase; 2) to evaluate the feasibility of adding a mixed enzyme system into the steep to reduce steeping time and enhance starch/protein separation; 3) to determine the effects of enzyme addition on the quality of starch; and 4) to investigate the mechanism by which enzymes affect wet milling performance.

MATERIALS AND METHODS

Preliminary Feasibility Study (Batch Steeping)

Steeping procedure

Corn was steeped following the method outlined by Krochta *et al*. (1981). A 300 g sample of corn and 600 ml of steeping solution were placed into a 1000 ml beaker. The beaker was immersed in a water bath set at 50°C. After the corn was steeped for the appropriate amount of time, the steep solution was drained off, measured, and analyzed for solids content. The corn was then subjected to the wet milling procedure described below (p. 44). Starch quality experiments were not performed on the starch separated in this preliminary study.

Corn was batch steeped under four conditions. The first treatment involved steeping for 48 hr. This treatment was used as the control to which the performance of the other three treatments were compared. The second treatment involved reducing the steeping time to 24 hrs in order to determine the effects of reducing the steeping period. These first two treatments used 0.2% SO_2 solution only. The third treatment utilized a mixed enzyme system in 0.2% SO_2 solution in a 24 hr steeping period. The fourth treatment, which also used a 24 hr steeping period, employed an experimental liquid enzyme preparation in 0.2% SO_2 solution. Use of this enzyme

preparation was confined to batch steeping only.

Steeping agents

The 0.2% SO_2 was prepared by dissolving sodium bisulfite in distilled water. The steeping solution containing the mixed enzyme system was composed of five enzymes chosen such that their optimal pH and temperature ranges fell within the typical pH range and temperature of steeping. Each enzyme, its source, optimum pH range, optimum temperature range, activity and manufacturer are listed in Table 4.

Some carbohydrase preparations considered for this study contained α-amylase activity which is an endo-splitting enzyme that hydrolyzes α-1,4-glycosidic bonds of starch to dextrins containing two to six glucose monomers. This activity would have imparted undesirable properties to the starch and decreased starch yields. Therefore, special care was taken in selecting carbohydrases with little or no α-amylase activity, especially β-glucanase which was derived from a special strain of Bacillus subtilis.

All five enzymes were added in equal weight concentration irrespective of their individual activities to dilute SO_2 solution. The total enzyme concentration of the steep solution was 1.25%.

The experimental enzyme preparation, labeled SP249, was obtained from Novo Industri (Wilton, CT). This liquid

Table 4. Enzymes in the mixed enzyme system, their sources, and their properties

Enzyme	Source	pH range	Temperature range (°C)	Activity (units/g)
Cellulase[a]	_Trichoderma viride_	4.0 - 6.0	50 - 60	1500
Hemicellulase[a]	_Aspergillus niger_	3.0 - 6.0	30 - 80	25
β-glucanase[b]	_Bacillus subtilis_	4.0 - 7.0	40 - 55	300
Pectinase[c]	_Aspergillus niger_	3.5 - 6.0	20 - 50	3000
Bromelain[a]	pineapple	4.0 - 5.5	45 - 55	3000

[a]Purchased from Sigma Chemical.

[b]Purchased as Pectinex 3XL from Novo Industri.

[c]Purchased as Ceremix 2XL from Novo Industri.

enzyme product, produced from a selected strain of _Aspergillus niger_, was designed for total degradation of plant cell walls and was recommended by Novo for use in corn steeping. The product contained the following activities:

Pectolytic

Polygalacturonase ... 9700 units/g

Pectinase .... 2035 units/g

SPS-ase .... 24 units/g

Cellulolytic

Cellulase .... 917 units/g

β-glucanase .... 152 units/g

Hemicellulolytic

Hemicellulase .... 288 units/g

Arabanase .... 98 units/g

Xylanase .... 86 units/g

Proteolytic

Protease .... 1120 units/g

Saccharifying

α-amylase .... unknown

glucoamylase .... unknown

The manufacturer claimed this product to be unique in that it contained a newly developed enzyme complex, SPS-ase, with the ability to decompose branched pectic polysaccharides (characterized from a soluble polysaccharide of soy beans) composed of galacturonic acid, rhamnose, xylose, fructose, arabinose, and galactose found in most vegetable tissues. Optimum pH and temperature conditions for this enzyme product were 3.5 - 5.5 and 40° - 50°C, respectively. The steeping solution for this treatment contained 1.25% of this enzyme preparation dissolved in 0.20% SO_2 solution.

Countercurrent Steeping

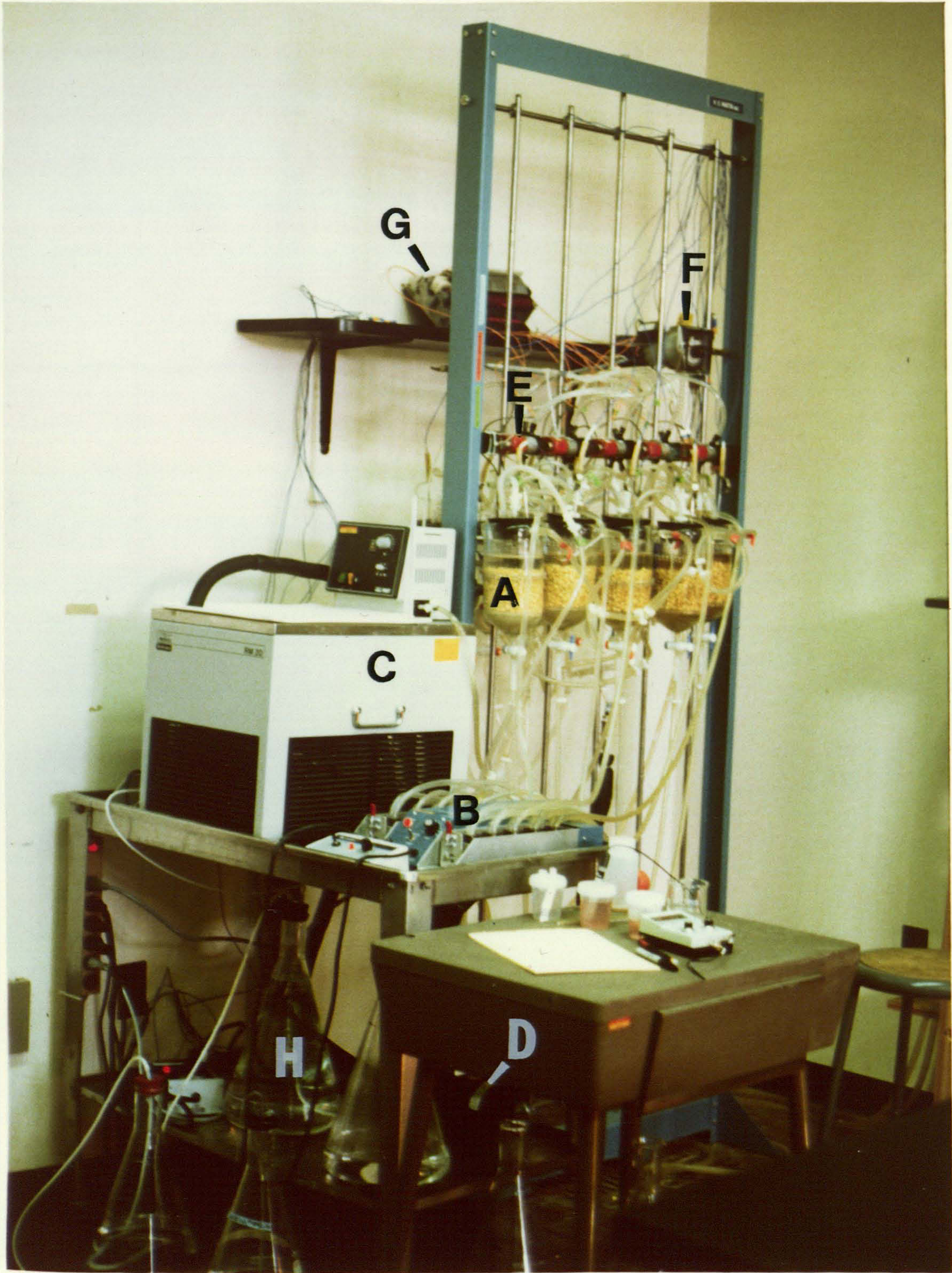
Materials and equipment

The countercurrent steeping system developed in this study (Figure 4) was based upon modifications to the continuous advance system used in commercial practice and described in detail by Watson (1984).

The steeping vessels (A) were 1200 ml jacketed, glass vessels (manufactured by the ISU Glass Shop) arranged in a battery of ten vessels. The mouth of each vessel was sealed with a No. 15 rubber stopper fitted with four teflon tubes. Three of the tubes were used as inlet tubes, one for recirculation within a vessel, another for incoming steepwater from the previous vessel, and the last for inflow of fresh steep solution. The fourth tube functioned as the overflow to the succeeding steep. The depth of this tube into the steep was carefully calibrated to 1050 ml for each vessel. The bottom of each vessel was drawn into a funnel-shape and was fitted with a teflon stopcock. A perforated plexiglass disk was placed in the bottom of the vessel to hold the corn in place. The temperature was maintained at 50 $\pm$ 2°C by circulating water through the vessel jackets with a water bath (C) coupled to a centrifugal pump (D).

Solution within a vessel was recirculated with a ten-channel peristaltic drive (B). The drive was equipped with

Figure 4. Laboratory countercurrent steeping system


G
F
E
A
C
B
H
D

ten pump heads capable of pumping 3.8 to 380 ml/min. Advancement through the battery was achieved by smaller peristaltic drive with a pump head (not shown) capable of pumping 0.2 to 21 ml/min.

The system for delivery of fresh steep solution was electrically automated. Inflow of steep solution into each vessel was actuated by a normally closed two-way solenoid valve (E) controlled by an Eagle Signal Controls (Wickes Mfg. Co.) Model HP59A6 timer (F) and a recycle module (Model HP50-103). The solution was advanced to succeeding steeps by a Model MT1916A62 electromechanical multi-circuit stepping programmer (G) from the same manufacturer.

All tubing connections were made with 5/16" I.D. Tygon tubing with the exception of the draw line which used 1/8" I.D. tubing. To provide easy removal of the steeps for cleaning, quick-disconnects were utilized on all five connections to each vessel.

<u>Countercurrent continuous advance steeping procedure</u>

Just before a vessel was to come on line, it was filled with 300 g of Pioneer Hybrid 3475 Yellow dent corn grown at an ISU test farm plot during 1987. The corn was forced-air dried to approximately 15.5% moisture at room temperature in one weeks time and screened to remove foreign material using a Carter dockage tester. Each steep was then inoculated with

5 ml of steep liquor obtained from Penford, Limited (Cedar Rapids, IA) in order to establish lactic acid producing species.

The steeping solution entered the system from a 6-L Erlenmeyer flask (H in Figure 4), was delivered to the top of the first steep, traveled down through the grain until the steepwater level rose to the bottom of the overflow tube, and overflowed into the top of the next steep. At the same time steep-water was recirculated within a steep through the larger peristaltic pump at the rate of 7 parts to one part new solution or carryover from a previous steep. The flux rate for recirculation was the same as used in industry. Advancement of solution through the steeps was achieved by positive pressure from the smaller peristaltic pump and by negative pressure from the larger peristaltic pump. For this reason each vessel was closed and liquid sealed. This sequence progressed until the sixth vessel was filled. At this time the stepswitch closed solenoid valve No. 1 and opened solenoid valve No. 2 to fresh steep solution. As a result, this brought vessel No. 7 on line and the corn in vessel No.1 was removed from the system. A maximum of six steeps were on line at once. This sequence of events continued in a circular fashion around the battery of ten vessels until equilibrium was reached. Equilibrium was considered to be the point at which the pH in the steeps leveled off. This usually required 96 hrs and discarding the

first 12 steeps.

The corn was steeped in three treatments of the same conditions used in the preliminary batch steeping study, the 48 hr control steep, the 24 hr shortened steep, and the 24 hr steep with addition of the mixed enzyme system. The treatment containing the experimental liquid enzyme preparation was eliminated from the countercurrent experiments since it was found to contain small amounts of saccharifying activities which may inflict detrimental effects on the starch.

In the countercurrent experiments, 0.20% SO_2 solution was prepared from 6% sulfurous acid rather than sodium bisulfite for the 48 and 24 hr steeping treatments. The pH of this solution was 2.2 compared to 4.5 for the batch steeping solution. Since the pH of this solution was low, sodium bisulfite was used as the source of SO_2 in the enzyme treatment steeping solution to avoid possible denaturation of the enzymes. The pH of the sodium bisulfite solution was adjusted to 3.40 with HCl before the enzymes were added.

The 48 hr steeping period required 8 hr to fill each vessel. During this filling period the steep water was also continuously recirculated within the steep. After 8 hrs, the steepwater overflowed into the succeeding steep and so proceeded throughout the steeps in 8 hr stages. After 48 hrs (at this point the sixth steep in a series was full) and every 8 hrs thereafter, a new steep came on line and the corn from

the first steep in the series was removed. The corn was either discarded if the system had not reached equilibrium or wet milled. The flow rates used in 48 hr steeping were patterned after industrial rates given by May (1987). During start-up the fresh solution flow rate was set at 1.7 ml/min, which corresponded to a control setting of 0.8 on the smaller peristaltic drive, until the sixth vessel was reached. At this time, the flow rate was increased to 2.2 ml/min (setting = 1.1) and continued at this rate for the rest of the run to generate steep liquor (246 ml/300 g batch of corn). The light steep liquor was collected and stored at 4°C. The recirculation flow rate was maintained at 15.5 ml/min throughout the run which corresponded to a setting of 4.0 on the larger peristaltic drive. Six replicates were collected for analysis.

Steeping for 24 hrs was run in basically the same way except the fill time was halved to 4 hrs and all the flow rates were doubled to 3.4 ml/min (small pump setting = 1.6) for the first five vessels, 4.4 ml/min (small pump setting = 2.2) for subsequent vessels, and 31.1 ml/min (large pump setting = 8.0) for the recirculation rate. The same amount of steep liquor (246 ml/300 g batch of corn) was collected. The 24 hr method was used for both the enzyme and non-enzyme treatments. Six replicates were collected per treatment for analysis.

Each lot of light steep liquor drawn was analyzed for pH, and SO_2, and solids contents. Samples were periodically drawn

from across the series of steeps to check on the condition of the run. The temperature and pH of every steep on line was measured at the end of each 4 or 8 hr stage to check the condition of the run and to assure the system was at equilibrium.

Wet Milling

Method

After steeping, the steepwater was drained from the steep and all of the steeped corn was removed. Wet milling of the corn began immediately.

The first step in the milling process was degermination which was accomplished by using a commercial Waring Blendor. Equal volumes of corn and water (approximately 100 ml) were measured into the 1200 ml blender jar and blended for 2.0 min at one-third speed. Speed was controlled with 115 V AC variac set at 33%. The variac was coupled to a laboratory timer. Each ground fraction was transferred to a 2000 ml beaker.

Germ separation was accomplished by adding an additional 100 ml of water to the beaker holding the corn slurry in order to facilitate germ flotation. The slurry was stirred and allowed to settle for a few minutes. Then the germs were skimmed from the surface along with a small amount of other corn parts with a wire screen (20 mesh). The germs were

removed from the screen with tweezers and placed in a 100 ml beaker. After germ separation was completed, the germs were washed three times with 100 ml of water each time. The wash water was returned to the degerminated corn slurry. The germs were dried, weighed for yield, and analyzed for moisture and protein content.

The degerminated corn slurry was prepared for fiber separation by reblending 400 ml of the slurry at a time in the Waring Blendor at full speed for 2.0 min. This step released the endosperm from the fiber and macerated the endosperm.

Fiber separation was performed by pouring approximately 200 ml of fully ground slurry onto a U.S. Standard No. 40 (8 in diameter) sieve stacked on top of a No. 200 sieve. The sieves were fitted to a pan for collection of mill starch. The sieving assembly was then shaken for 5.0 min on a Ro-Tap Testing Sieve Shaker with tapping. The fraction retained on top of the No. 40 sieve was termed "coarse fiber", the fraction remaining on the No. 200 sieve was called "fine fiber", and the material that passed through the sieves was termed "mill starch". The fractions were transferred to beakers with a rubber scraper. The fiber fractions were washed of starch and protein by transferring the fraction onto a Spectra/Mesh nylon cloth (Fisher Scientific) woven with 52 micron openings and then by dunking the fiber-containing cloth into a series of three beakers containing 400 ml of water each for 1.0 min per

beaker. As much water as possible was squeezed from the cloth into the last beaker after the washing cycle was completed. The fiber was removed from the cloth with a rubber scraper, dried, and analyzed for yield, moisture, and protein content. The wash water was combined with mill starch pan components in a 4000 ml beaker, stirred, and allowed to settle for 24 hrs at 4°C.

The last step in the milling procedure involved starch and protein separation. Separation was initiated by decanting the majority of the wash water into another 4000 ml beaker leaving approximately 800 ml of mill starch and water. This mill starch slurry was transferred to a 1000 ml beaker, stirred vigorously, and allowed to settle for another 24 hrs at 4°C. The smaller surface area of the 1000 ml beaker greatly improved the separation of the starch and protein. After 24 hrs, as much water as possible was decanted into the 4000 ml wash water beaker. The wash water was returned to cold storage and later analyzed for solids and protein contents. The protein was carefully slurried, poured, and rinsed from the starch. The unpurified gluten fraction was then transferred into 250 ml plastic centrifuge bottles and centrifuged for 30 min at 6000 G's. The water was decanted and the bottles were laid on their sides after centrifugation to prevent the separated fractions from sliding together. The protein layer was carefully scraped off the surface of the starch with a

Scoopula. Between the two layers was an off-white layer composed of protein-bound starch. This layer was scraped off and collected as "inseparables". The bottom-most layer of starch was slurried and combined with the starch in the 1000 ml beaker separated by gravity in the cold room. The inseparable layer was then reslurried, centrifuged, and separated as described above. With this variety of corn, no more the two centrifugations were required.

The three fractions were slurried and poured into shallow pans in preparation for drying. A 22.9 cm (9 in) pie pan worked well for the starch while a 12.7 cm (5 in) sieve receiving pan was used for the gluten and inseparable fractions.

Special care was taken throughout the entire milling procedure to minimize spillage. Any spillage was returned to its respective container to minimize yield losses.

Analyses of the wet milled fraction

Moisture removal: All fractions, except starch, were dried for 24 hrs in an oven at 65°C followed by 8 hrs in a vacuum oven at 65°C. Starch was dried in an oven at 40°C for 48 hrs to minimize damage to the starch.

Moisture content: The Vacuum Oven Method 44 - 40 of the American Association of Cereal Chemists (AACC, 1982) was used to determine the moisture content of the corn and dried

fractions.

Solids content: A 10 ml sample of steep water or starch wash water was precisely transferred into a tared aluminum weighing pan. The pans were dried at 65°C for 24 hrs and then dried to a constant weight in a vacuum oven at 65°C. Percent total solids in the steep water were computed as the ratio of dry matter to the initial 10 ml of liquid sample weight times 100. The total solids in the starch wash water was computed as the product of the ratio of dry matter to the initial 10 ml of liquid sample weight times the total of starch wash water.

Fraction yield: The fraction yields were determined on a dry material basis. Yield percentages were calculated as the weight of the fraction after moisture removal minus the moisture content divided by the dry basis weight of the total of fractions recovered.

Protein content: Percent nitrogen in each fraction was determined by a macro-Kjeldahl method with a Kjeltec Digestion System 6 digester and System 1002 Distilling Unit (Tecator, Inc.). The amount of sample analyzed was adjusted to use between 10 and 50 ml of titrant (0.5 g to 2.0 g of sample). A factor of 6.25 was multiplied by the % N to calculate the % protein.

Sulfur dioxide content: SO_2 was determined by titration with standard iodine-potassium iodide solution adjusted to be equivalent to 0.001 g SO_2 per ml. Soluble

starch was used as the indicator (Watson et al., 1955a).

Starch Quality Evaluation

Viscosity

The viscosity of starch slurries from the three steeping treatments were charted during pasting, cooking, and cooling with a Brabender Viscoamylograph (C.S. Brabender Instruments, Inc.). The calibration of the instrument was checked with calibration starch obtained from the manufacturer and found to be within specifications.

The starch was analyzed according the method of Mazurs *et al*. (1957). This procedure involved preparation of an 8% starch solution composed of 40 g of starch (on a dry basis, by prior moisture determination) and 460 g of distilled water. The starch was slurried in approximately 3/4 of the water and transferred to the cup of the instrument. The remaining 1/4 of water was used to rinse the beaker. The slurry was heated to 95°C at the rate of 1.5°C per min, maintained at that temperature for 15 min, and then cooled at a rate of 1.5°C per min to 50°C. Three runs were performed for each sample of starch. Five significant points of interest were observed along the viscosity curve (Zobel, 1978):

A. Peak pasting temperature, described as the point at which there was an increase of ten Brabender

units from onset of gelatinization.

B. Peak viscosity, irrespective of the temperature at which the pasting peak was attained. This was important because this stage must be cooked through to obtain a usable starch paste.

C. The viscosity when the paste reached a temperature of 95°C. The relationship of this value to the peak viscosity reflected the ease of cooking the starch.

D. Viscosity after the paste was cooked for 15 min at 95°C. This indicated the stability or breakdown of the paste during cooking.

E. The viscosity of the cooked paste during cooling was a measure of the set-back produced by cooling.

Thermal properties

Differential Scanning Calorimetry (DSC) was used to study the thermal properties of starch when heated with water. Information such as onset, peak, and enthalpy of gelation were computed.

DSC measurements were made with a Perkin-Elmer DSC-7 equipped with a Thermal Analysis Data Station. Four-mg samples of starch were weighed into aluminum sample pans with 8 µl of water. The pan was hermetically sealed and placed in the

instrument. A pan containing only 8 µl of water was used as a reference. Samples were heated at a rate of 10°C per min from 30°C to 120°C. Enthalpy, onset, and peak temperature were computed automatically. All samples were run in triplicate.

Colorimetry

Color measurements of starch from all treatments were observed with a Labscan 5100 spectrocolorimeter (Hunter Associates Laboratory, Inc.). Starch samples were loaded into aluminum weighing pans and filled such that no part of the inner metal surface was exposed to light. A total of three measurements were made per starch sample.

The Hunter *L*, *a*, *b*, opponent color scale was used and calculated relative to the Commission Internationale de l'Eclairage (CIE) 1964 10° Standard Observer. The opponent color scales gave measurements of color in units of approximate visual uniformity. Thus, *L* measured lightness and varied from 100 for perfect white to zero for black. The chromaticity dimensions (*a* and *b*) gave designations of color such that *a* measured redness when plus, gray when zero, and greenness when minus and *b* measured yellowness when plus, gray when zero, and blueness when minus. In the case of starch the degree of lightness (*L*) and the absence of yellowness (+*b*) were important.

Diffusivity

An experiment was performed to compare the rates of water absorption of corn steeped in the enzyme mixture steep solution versus steepwater containing only 0.2% SO_2. The two treatments were randomly assigned to six vessels (each treatment was replicated three times). About 300 g of corn used in the countercurrent study and 820 ml of steep solution were added into a vessel. The plumbing to the vessels was modified to a circulating batch configuration.

Approximately 5 g of steeped corn was removed for moisture analysis every 6.0 min (0.1 hr) for the first hour, every 0.5 hr thereafter until the sixth hour, and at 7, 8, 9, 10, 12, 16, 20, and 24 hrs. The samples were blotted dry with a tissue to remove surface droplets and weighed into tared aluminum pans. Moisture content of the corn, which was predried in an oven for 24 hrs at 65°C, was determined by the Vacuum Oven Method 44 - 40 of the AACC (1982). The initial drying step was required to prevent overloading of the vacuum system.

A Basic computer program developed by Hsu (1984) was used to calculate diffusivity constants. Moisture at a given time, maximum absorption, and the average radius of a single kernel were the parameters entered. The average radius was determined from the volume of three lots of 100 unbroken kernels. The

volume of the kernels was calculated by a Beckmann Model 930 Air Comparison Pycnometer. The equation $4/3\pi r^3$ was solved for r and an average value for the radius of a single kernel was computed by dividing by 100.

Microscopic Examination of Steeped Kernels

It has been suggested that the carbohydrases have the greatest effect on the cross cell and aleurone layers of the corn because of their granular nature (Wolf et al., 1952a). Wood and McRae (1978) reported that these granular areas in cellulose containing structures are most susceptible to carbohydrase degradation. Therefore, the fiber of steeped kernels was microscopically examined for signs of enzyme degradation by following a method described by Wagoner (1948).

Several kernels of average size and shape were selected from steeped corn samples from each treatment. A cross section of each kernel was obtained by slicing about 1 mm below the crown and approximately 1 mm above the scutellum with a new razor blade. Frozen sections were made with a CTF sliding microtome-cryostat (International Equipment Co.) at -10°C. A trimmed kernel was placed in a drop of IEC Cryoform solution on the freezing stage and admitted to the freezing chamber. As the freezing progressed, more solution was placed around the kernel until it was completely encased. After 15 min,

successive 40 micron slices were made until satisfactory sections were collected. Best results were obtained when a drop of water was placed on the upper knife edge as the blade was slowly pushed across the kernel (Watson and Hirata, 1954) so that the section thawed as it was cut. Intact sections were removed with a camel's hair brush and placed in distilled water on a microscope slide. A drop of 0.05 N iodine solution was placed on it, allowed to stand for 3 min, and then washed off. Cross sections were more easily observed under the microscope when the starch was removed. This was accomplished by placing several drops of 75% chloral hydrate on the stained section and carefully brushing out the starch granules with a fine-hair brush. The section was then washed and a few drops of 0.25% thionine solution, adjusted to a purple color with ammonia, were placed on it. After 10 min the solution was rinsed off. A drop of glycerin was added to preserve the sample for a few days.

Photomicrographs were taken with a Spencer Model 635 35mm camera attached to a Series 4 Microstar trinocular microscope (American Optical Co.). Kodak Ektachrome ASA 160 film with a Kelvin rating of 3200 was used for exposure. The built-in base illuminator and variable transformer (set at 6.5) were used as the light source. Exposure settings of 1/5, 1/10, and 1/25 provided the best results. The sections were magnified through 3.5x and 10x objectives producing total magnifications of 8.75

and 25, respectively (the photographic eyepiece magnified the objective image 2.5 times).

Separation of Fibrous Layers

This macroscopic method was also performed to examine the effects of the carbohydrases on the fibrous layers of steeped kernels. Fifty steeped corn kernels were randomly selected from each treatment. The outer layer (or layers) of the kernel was (were) removed by firmly grasping the kernel between two fingers at the crown while pinching the tip cap with two fingers of the other hand and slowly pulling away a thin strip of fiber. The sample was categorized as "attached" if the seed coat was removed along with the pericarp or "detached" if the pericarp was removed alone.

RESULTS AND DISCUSSION

Preliminary Feasibility Study (Batch steeping)

This preliminary study was performed to evaluate the gross feasibility of enzyme addition to improve the wet millability of corn in a shortened steeping period. The effects of four steeping conditions were evaluated by randomly assigning the four treatments to 300 g lots of corn. Six samples per treatment were collected and subjected to wet milling. The values for fraction yield and protein content were analyzed for variance and the means compared by Duncan's test for significance at the 5% probability level. The results are given in Table 5. The 48 hr treatment was used as the standard by which the other three treatments were compared. No difficulties in wet milling separation were encountered.

Evidence of insufficient steeping was visually observed during the wet milling of 24 hr steeped corn. The first indication was noticed in the degermination step where fiber and pieces of endosperm remained attached to the germ. The second was found in the fiber fraction where many small pieces of horny endosperm remained on the sieves even after grinding at full speed in the Waring Blendor. The 24 hr yield and protein content fraction results were found to be significantly inferior to the 48 hr steeped corn results. This suggests

Table 5. Yield and protein composition of mill fractions from batch steeped corn

Fraction	48 hr steeping control	24 hr steeping: Control	24 hr steeping: Mixed enzyme system	24 hr steeping: Exp. enzyme product
Germ				
Fraction yield, %	6.62[a]	7.31[b]	6.67[a]	6.71[a]
Protein, %	17.6[a]	18.5[b]	17.4[b]	18.6[b]
Total fiber				
Fraction yield, %	19.20[ab]	25.74[c]	17.63[b]	20.12[a]
Protein, %	11.6[a]	13.1[b]	11.8[a]	12.8[b]
Starch				
Fraction yield, %	58.44[a]	48.73[b]	57.72[a]	54.87[c]
Protein, %	0.56[ab]	1.20[c]	0.52[b]	0.63[a]
Gluten				
Fraction yield, %	5.16[a]	5.92[b]	5.85[b]	5.41[a]
Protein, %	56.0[a]	42.8[b]	57.4[a]	56.8[a]
Inseparables				
Fraction yield, %	3.73[a]	7.07[b]	4.51[a]	5.33[c]
Protein, %	7.5[a]	10.3[b]	6.1[c]	8.6[a]
Solubles[d]				
Fraction yield, %	7.22[a]	5.88[b]	7.62[a]	7.55[a]

[abc]Values within a row with the same letter superscript are not significantly different.

[d]Composite of starch wash water and steep solubles.

that a shortened steeping period significantly affected wet milling performance. Most noticeable were the increased fiber yield, decreased starch yield, increased protein content in the

starch fraction, decreased protein content of gluten, increased inseparables yield, and increased protein content of the inseparables fraction. These differences were attributed to incomplete reduction of the protein matrix by SO_2.

Yield and protein content values for both enzyme treatments in most fractions were not significantly different from the 48 hr values and were significantly superior to the 24 hr values. This provided evidence that both enzyme treatments improved the wet millability of corn in a shortened steeping period. In fact, evidence that these treatments produced slightly better results (but not significant) were found in decreased protein content of starch by the mixed enzyme system treatment and increased protein content of gluten in both enzyme treatments. This was attributed to the enzymes enhancing starch and protein separation.

During wet milling, none of the indications of insufficient steeping observed during processing of the 24 hr treated samples were noticed in either enzyme treatment. However, two differences between the enzyme and nonenzyme treatments were perceived. The first difference was a petroleum-like odor, similar to butyl alcohol, which emanated from the steep solution after approximately 36 hrs. No attempt was made to identify this substance which was most likely a by-product of enzymatic hydrolysis. The second dissimilarity occurred in the outward appearance of the corn kernels where a

slight grayish-black tint was observed in the kernels after steeping was complete. These color differences can be seen in Figure 8 later in this study. Although the experimental enzyme product treatment was excluded from this photo, the results observed were similar to the mixed enzyme system treated corn.

Even though there were significant differences in eight of eleven values for the two enzyme treatments, direct comparisons can not be made since the activities and sources of the individual enzymes in the two mixtures were different. However, both enzyme preparations improved the wet millability of corn in a shortened steeping period, therefore the study was continued with countercurrent steeping.

Countercurrent Steeping

Preliminary work

It was found during the preliminary batch steeping study that the wet milling of corn samples required a substantial time investment. Therefore, one of the enzyme treatments was removed from the countercurrent study. The experimental enzyme product, Novo's SP249, was the logical choice since it contains saccharifying activities which may inflict detrimental effects on the starch and was less effective than the experimental mixture. It was also determined in the batch steeping study that in the case of future investigations it would be

convenient if the composition of the enzyme mixture was precisely known and could be manipulated. All of the enzymatic activities in the experimental enzyme product were derived from one selected strain of Aspergillus niger in a preprepared mixture. Adjustment of the experimental enzyme product would be impossible in the laboratory. Since only one enzyme treatment will be used in this portion of the study, the mixed enzyme system treatment will be referred to as the enzyme treatment from this point on.

During the calibration of the countercurrent system, it was discovered that the corn expanded more than anticipated in the steeps leaving approximately one-half of the corn uncovered with the peristaltic pump set at the flow rate initially calculated (0.8 ml/min). In industry, the enormous weight of the corn in the steeps prevent the corn from expanding as much and provides the opportunity for using smaller amounts of solution (Watson, 1984). The only solution to this dilemma was to increase the inflow rate from 0.8 ml/min as suggested by May (1987) to 1.7 m/min. Therefore, all of the system parameters (pH, dissolved solids, and SO_2) were expected to differ by approximately a factor of one-half from those observed in industry. Since this study involved comparisons between treatments, exact simulation was not considered to be critical.

The countercurrent system required very little maintenance

and only a small amount of manual attention during operation. This manual attention involved occasional slight adjustments in the input flow rate, removal of steeped corn, and collection of steep liquor.

Steepwater pH

The goal of simulating a commercial steeping pH profile (pH 3.8 to 4.1) was not precisely accomplished in the 48 hr experiment (Table 6) because of the adjustment in flow rate mentioned previously. For the first three steeps the pH remained relatively stable due to the acidic nature of the SO_2 solution. As the SO_2 was depleted however, the pH began to rise from steep 4 through steep 6. This was partially due to the increased inflow rate of steep solution diluting the buffering effect of remaining SO_2 and any lactic acid produced.

Another dilemma was encountered during establishment of the pH profile. To maintain the pH values of 3.5 to 4.7 the pH of fresh steeping solution had to be kept at 2.2, compared to fresh commercial steepwater which is introduced at pH 3.4. Watson et al. (1951) were able to sustain lactic acid fermentation with steep solution having a pH as low as 3.1. However, with a pH as low as 2.2, it was speculated that most of the Lactobacilli present may have had their growth inhibited. This may have also contributed to the rise in pH later in the steeping system. Despite these circumstances, the

Table 6. Steepwater pH values in individual steeps of a laboratory countercurrent steeping system

Treatment	Steeping time (hrs)	Steep number						
		F[a]	1	2	3	4	5	6[b]
Control	48	2.20	3.50	4.02	4.10	4.34	4.80	4.74
Control	24	2.20	3.11	3.50	4.05	4.47	4.71	4.69
Enzyme	24	3.40	4.70	4.85	4.95	4.95	4.75	4.65

[a]F denotes fresh steep solution.

[b]Steep no. 6 contains the newest corn.

wet millability of the corn subjected to the 48 hr treatment and the quality of starch were not adversely affected.

The outcome for the 24 hr treatment was as anticipated. The pH across the system was slightly lower due to the fact that it was not possible for the corn to absorb as much SO_2 in the shorter steeping period.

A relatively consistent pH profile was produced in the system during the enzyme steeping experiment contrary to the conditions produced during 48 hr steeping where the pH steadily increased. This situation may be attributed to a pH environment that was more favorable for the growth of Lactobacilli than the environment produced in the 48 hr experiment. Wahl (1969) determined that the greatest amount of

Lactobacilli activity occurs at about pH 5. An increase in the amount of substrate available by enzymatic hydrolysis for conversion to lactic acid by Lactobacilli may also have contributed to this buffering effect.

The pH profile for the enzyme treatment was higher overall than the pH profile produced in the 48 hr treatment because of the difference in the source of SO_2 used. The source of SO_2 for the 48 hr treatment solution (pH 2.2) was sulfurous acid while the enzyme treatment solution (pH 3.4) used sodium bisulfite. Although the pH values were expected to be about one pH value lower across the system, decreasing the pH of the enzyme steeping solution was not attempted for fear of denaturing the enzymes. Despite the higher pH, the system operated within the activity ranges of the enzymes.

Steepwater dissolved solids content

The dissolved solids content across the system for the 48 hr treatment (Table 7) was approximately one-half as concentrated as the dissolved solids content found across a commercial system (see Figure 3) as expected. This was largely attributed to the higher feed rate of steeping solution.

At each stage across the system, approximately one-half as much dissolved solids accumulated in the steepwater of the 24 hr treatment as in the steepwater of the 48 hr treatment. Since the steepwater remained in contact with the corn for

Table 7. Dissolved solids composition of steepwater in individual steeps of a laboratory countercurrent steeping system

Treatment	Steeping time (hrs)	Steep number						
		F[a]	1	2	3	4	5	6[b]
Control	48	0	0.63	1.21	1.81	2.38	3.01	3.30
Control	24	0	0.47	0.76	1.15	1.39	1.48	1.55
Enzyme	24	0	0.74	1.43	2.13	2.81	3.55	3.89

[a]F denotes fresh steep solution.

[b]Steep no. 6 contains the newest corn.

one-half as long, this was also anticipated.

The amount of dissolved solids for the enzyme treatment were higher than the 48 hr levels at each stage suggesting some cell breakdown attributed to enzyme action. In fact, the concentration of enzymes may have been too high and might be reduced in future studies to bring the level of dissolved solids down into the range of those generated by the 48 hr treatment. The amount of dissolved enzyme was subtracted from the total amount of dissolved solids during steepwater dissolved solids determination.

<u>Steepwater sulfur dioxide content</u>

The steepwater SO_2 level in individual steeps for the 48 hr and enzyme treatments listed in Table 8 approximately followed the trend shown in Figure 3 for a commercial system, after compensation for the dilution factor. For this trend to have occurred in the enzyme steeps, the enzymes must have facilitated the penetration of SO_2 into the kernels in the shortened steeping period.

The levels were higher in each 24 hr steep as anticipated. Again this was a result of increased flow rate and a decreased steeping period reducing the amount of SO_2 absorbed.

Table 8. Sulfur dioxide levels in individual steeps in a laboratory countercurrent steeping system

Treatment	Steeping time (hrs)	Steep number						
		F[a]	1	2	3	4	5	6[b]
Control	48	0.20	0.13	0.12	0.11	0.10	0.08	0.06
Control	24	0.20	0.17	0.17	0.14	0.12	0.12	0.11
Enzyme	24	0.20	0.14	0.12	0.10	0.10	0.09	0.08

[a]F denotes fresh steep solution.

[b]Steep no. 6 contains the newest corn.

Wet milling analysis of countercurrent steeped corn

The effects of the 48 hr, 24 hr, and enzyme treatments were evaluated by randomly assigning 300 g lots of corn to vessels for steeping. The observations for fraction yield and protein content obtained by wet milling were then analyzed for variance and the means compared by Duncan's test for significance at the 5% probability level. The mean values are given in Table 9. The 48 hr treatment was again used as the control by which the other two treatments were to be compared. No difficulties in wet milling separation were observed with the 48 hr treatment.

Similar evidence of insufficient steeping was visually observed during the wet milling of corn continuously steeped for 24 hr as was observed in the preliminary batch steeping study. When fraction yield and protein content for each fraction of the 24 hr and 48 hr treatments were compared, the values for the 24 hr treatment were found to be significantly inferior indicating incomplete steeping in the shortened steeping period. Most recognizable differences were the increased protein percentage of the germ, decreased starch yield with increased protein content, decreased protein percentage in the gluten, and increased inseparables yield.

All of the mean fraction values for the enzyme treatment were also found to be significantly superior to all of the 24 hr mean values. This indicated that enzyme addition improved

Table 9. Yield and protein composition of mill fractions from countercurrent steeped corn

Fraction	48 hr steeping control	24 hr steeping	
		Control	Enzyme
Germ			
Fraction yield, %	6.72[a]	7.45[b]	6.72[a]
Protein, %	14.6[a]	17.2[b]	15.2[a]
Total fiber			
Fraction yield, %	10.65[a]	11.50[b]	12.51[c]
Protein, %	9.9[a]	11.1[b]	7.9[c]
Starch			
Fraction yield, %	64.94[a]	56.95[b]	63.98[c]
Protein, %	0.42[a]	0.82[b]	0.33[c]
Gluten			
Fraction yield, %	5.57[a]	6.34[b]	5.80[a]
Protein, %	47.6[a]	44.4[b]	50.1[c]
Inseparables			
Fraction yield, %	4.44[a]	10.78[b]	2.35[c]
Protein, %	3.2[a]	1.8[b]	4.5[c]
Solubles[d]			
Fraction yield, %	7.68[a]	6.98[b]	8.64[c]

[abc]Values within a row with the same superscript are not significantly different.

[d]Composite of starch wash solubles and solubles lost in steep solution.

the wet millability of corn in a shortened countercurrent steeping period. No significant differences were found between the germ yield, the protein percentage of the germ, and the

gluten yield results from the enzyme and 48 hr control. However, significant differences were found between the rest of the fraction yield and protein percentage values. These differences were a consequence of enhanced protein and starch separation through enzyme addition which produced significantly superior results over the 48 hr control such as increased fiber yield with decreased protein content, decreased protein content in starch, increased protein content in gluten, and decreased inseparables yield. Even though the percentage of protein in the starch fraction of the enzyme treatment was lower than the 48 hr control, the starch yield was approximately 1% less. Most of this starch was lost during steeping and was observed in the bottom of the steeping vessel after the steeped corn was removed. This starch loss was attributed to enzyme action which facilitated the release of starch into the steepwater, especially from cracked kernels, contributing to the significant increase in the enzyme treatment solubles yield.

Comparison of laboratory wet milling procedures

Wet milling methods are evaluated based on the ability to maximize fraction yield and purity, which is represented by the percentage of protein in the fraction. A high starch yield, a low percentage of protein in the starch fraction, and a high level of protein in the gluten fraction are considered to be

the best indicators of method performance. Therefore, wet milling results from industry, Watson's laboratory procedure, and 48 hr batch and countercurrent steeped corn produced by this investigation are given in Table 10 for purposes of comparison.

When the batch steeping results produced by this investigation were compared with the results from Watson's laboratory procedure, both methods generated starches with nearly identical protein content, however, there was a large discrepancy in the fraction yields. Based upon the higher purity of the fractions produced in this study, it is unlikely that the difference can be attributed to starch lost to other fractions but rather to differences in original kernel composition. The large dissimilarity in fiber yield was also attributed to variation in original composition. Despite these discrepancies, the techniques used in this study compared favorably with the previously published results.

The countercurrent steeping results produced by this investigation were more typical of commercial experience than either found in Watson's laboratory procedure or our own simulation of Watson's procedure. The most noticeable difference was the superior purity of the fractions, in particular the starch and gluten fractions. Thus, laboratory countercurrent steeping was found to be more effective than laboratory batch steeping.

Table 10. Yield and protein composition of mill fractions produced from industrial processing and laboratory procedures

Fraction	Industrial processing[a]	Laboratory procedures: Watson's batch steeping[a]	Batch steeping (present study)	Counter-current steeping
Germ				
Fraction yield, %	7.5	6.2	6.6	6.7
Protein, %	12.0	23.7	17.6	14.6
Fiber				
Fraction yield, %	11.5	12.5	19.2	10.7
Protein, %	12.0	14.7	11.6	9.9
Starch				
Fraction yield, %	67.5	65.4	58.4	64.9
Protein, %	0.30	0.54	0.56	0.42
Gluten				
Fraction yield, %	5.8	8.1	5.2	5.6
Protein, %	65.8	42.9	56.0	47.6
Squeegee[b]				
Fraction yield, %	...	1.4	3.7	4.4
Protein, %	...	20.8	7.5	3.2
Solubles				
Fraction yield, %	7.5	7.1	7.2	7.7

[a]Results taken from Anderson and Watson (1982).

[b]Equivalent to inseparables fraction. This fraction is not produced in industry.

Starch Quality Evaluation

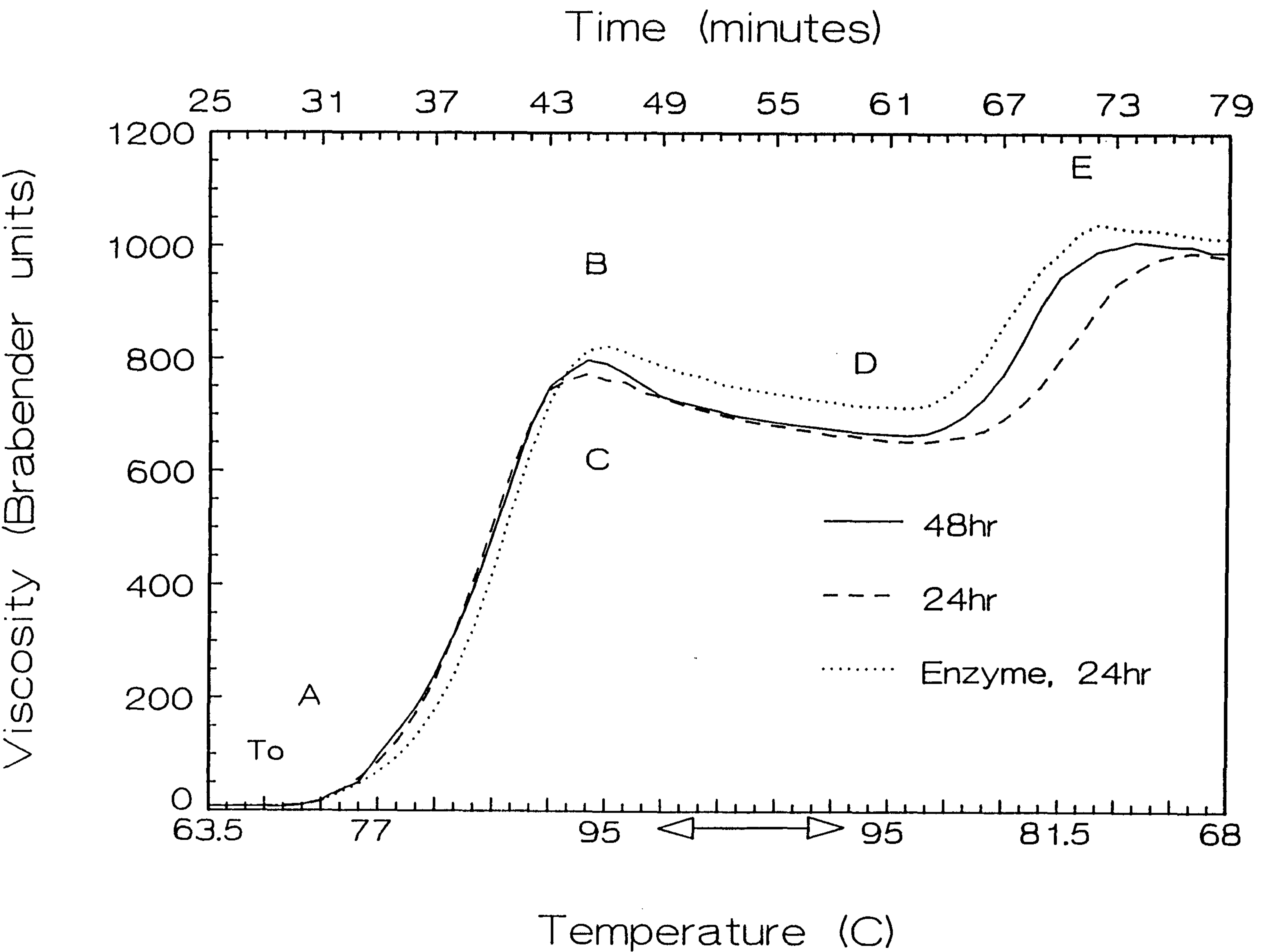
Viscosity

It is important that any enzyme treatment not alter thermal properties of starch. The pasting behavior of starch, as a function of temperature and time measured in the Brabender Viscoamylograph for the 48 hr, 24 hr, and enzyme treatments are shown in Figure 5.

The starch from all three treatments exhibited typical pasting behavior throughout the curve which was characteristic of corn starch obtained by wet milling normal dent corn (Greenwood, 1978). Actual values for the five points of interest and another point of interest not generally recognized on the Brabender curve, onset temperature of gelatinization (To), are given in Table 11. To is useful in comparison of starch evaluation methods (Greenwood, 1978).

There were no significant differences between any of the points of interest along the Brabender curves when comparing the 48 hr and enzyme treatments or the 48 hr and 24 hr treatments together. However, there was enough difference between the 24 hr and enzyme treatments at all of the points of interest to be considered significant, especially in the area of set-back (E). The level of protein contamination (see Table 9) was believed to have been the major cause of these differences.

Figure 5. Brabender viscoamylograph curves of laboratory-isolated starches. To = onset temperature; A = peak pasting temperature; B = peak viscosity; C = peak viscosity at 95°C; D = extent of breakdown after cooking at 95°C for 15 min; E = amount of set-back



Time (minutes)
25
31
37
43
49
55
61
67
73
79
Viscosity (Brabender units)
1200
1000
800
600
400
200
0
E
B
D
C
A
To
48hr
24hr
Enzyme, 24hr
63.5
77
95
95
81.5
68
Temperature (C)

Table 11. Values for significant points of interest along Brabender viscoamylograph curves of laboratory-isolated starches

Treatment	Steeping time (hrs)	Points of interest[a]					
		To(°C)	A(°C)	B(°C)	C(°C)	D(°C)	E[b] (Bu[c]/min)
Control	48	69.5[d]	72.0[d]	798[de]	791[de]	664[de]	1010[de]/74
Control	24	68.0[d]	73.0[d]	774[d]	761[d]	652[d]	990[d]/77
Enzyme	24	68.0[d]	72.5[d]	823[e]	823[e]	710[e]	1040[e]/72

[a]To denotes onset temperature; A, peak pasting temperature; B, peak viscosity; C, peak viscosity at 95°C; D, extent of breakdown after cooking at 95°C for 15 min; E, amount of set-back.

[b]Maximum value of set-back/time at which the maximum value occurred.

[c]Bu denotes Brabender units.

[de]Values with the same letter superscript within a column are not significantly different.

Osman (1967) observed that contaminants such as protein have been known to influence starch gelatinization through competition for available water. She also observed interference with molecular forces between starch components and disruption of the aggregation of amylose. Effects such as increased pasting temperature (A), decreased peak viscosity (B), decreased stability during cooking (D), and decreased and

delayed set-back (E) were recognized. Most of these effects were observed in the starch from the 24 hr treatment.

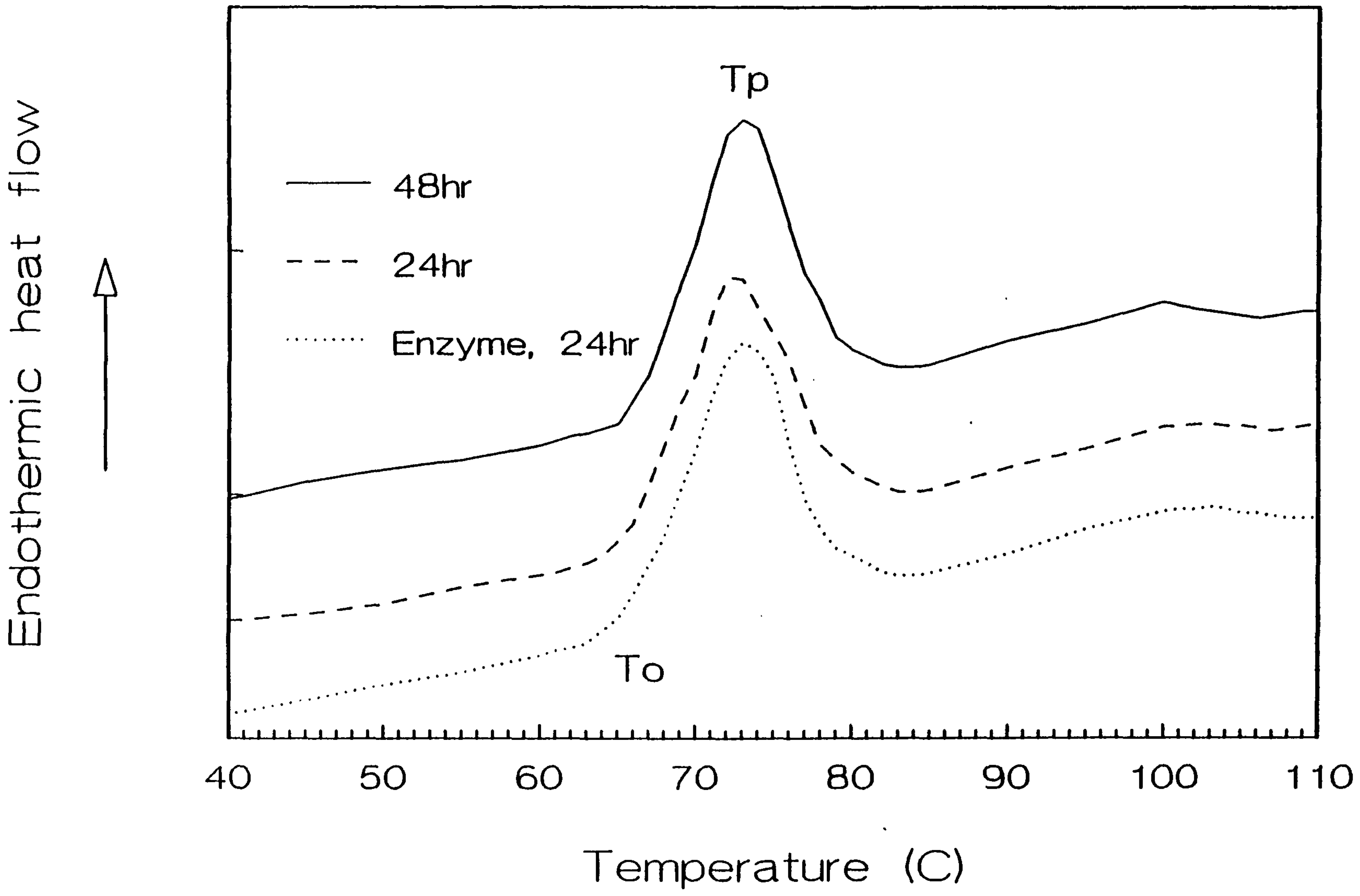
Thermal properties

Gelatinization characteristics of starch determined by differential scanning calorimetry (DSC) for the 48 hr, 24 hr, and enzyme treatments are displayed in Figure 6. Differences in peak maxima of treatment thermograms in Figure 6 (i.e., 48 hr > 24 hr > enzyme) were not significantly different. Rather, comparisons of thermogram shape and area under the peak served as a means of evaluation.

The thermograms from all three treatments were similar to those obtained by Krueger et al. (1987) for laboratory isolated corn starch. The actual values for the onset temperature of gelatinization (To), peak gelatinization temperature (Tp), and enthalpy of gelatinization (ΔH) are given in Table 12.

Although this experiment was performed to determine differences between treatment thermograms, of which none were significant, other interesting observations were made. The To calculated by DSC was determined to occur earlier than that determined by the Brabender instrument in accordance with results described by Zobel (1978). However, the peak pasting temperatures calculated by DSC (Tp) were not significantly different from the peak pasting temperatures taken from the Brabender curves (designated "A"). The effects of annealing on

Figure 6. Differential scanning calorimetry thermograms of laboratory-isolated starches. To = onset temperature; Tp = peak pasting temperature; ΔH = the area under the peak



Tp
48hr
24hr
Enzyme, 24hr
To
Endothermic heat flow
40
50
60
70
80
90
100
110
Temperature (C)

Table 12. DSC thermogram values of laboratory-isolated starches

Treatment	Steeping time (hrs)	Thermographic values[a]		
		To (°C)	Tp (°C)	ΔH(calories/g)
Control	48	67.2	73.2	3.00
Control	24	66.5	73.0	3.00
Enzyme	24	66.8	73.2	3.11

[a]None of the values were significantly different within a column.

the gelatinization behavior of corn starch observed by Krueger <u>et al</u>. (1987) was also apparent in the starches examined in this experiment. Consequences, such as narrower gelatinization range, higher Tp, and higher ΔH, indicated internal structural changes in the starch granules as a result of the wet milling process in comparison to raw extracted starch were also observed. Effects such as decreased ΔH and increased Tp as a result of protein contamination in starch reported by Eliasson (1983) were not observed here in the 24 hr treatment thermograms. The most likely cause to this situation was the small, unrepresentative amount (4 mg) of starch sample used for DSC analysis. This would also explain why the effects of protein contamination were observed in the Brabender analysis

where a larger sample of starch (40 g) was used.

Colorimetry

The chromacity dimensions for the degree of lightness (L) and the intensity of yellowness (+b) for the starch samples from the 48 hr, 24 hr, and enzyme treatments are given in Table 13. The 48 hr and enzyme treatment L values were significantly closer to a perfect white value of 100 than the L value for the 24 hr treatment suggesting that the 48 hr control starch samples contained a greater percentage of starch. This suggestion was supported by the fact that the starch from the 24 hr treatment produced a significantly higher level of yellowness (+b), derived from the carotenoid pigments associated with corn protein, than the other two treatments.

Table 13. Chromacity values of laboratory-isolated starches

Treatment	Steeping time (hrs)	Chromacity values	
		L	+b
Control	48	97.2[a]	5.59[a]
Control	24	96.8[b]	6.56[b]
Enzyme	24	97.2[a]	5.89[a]

[ab]Values with the same letter superscript within a column are not significantly different.

Diffusivity

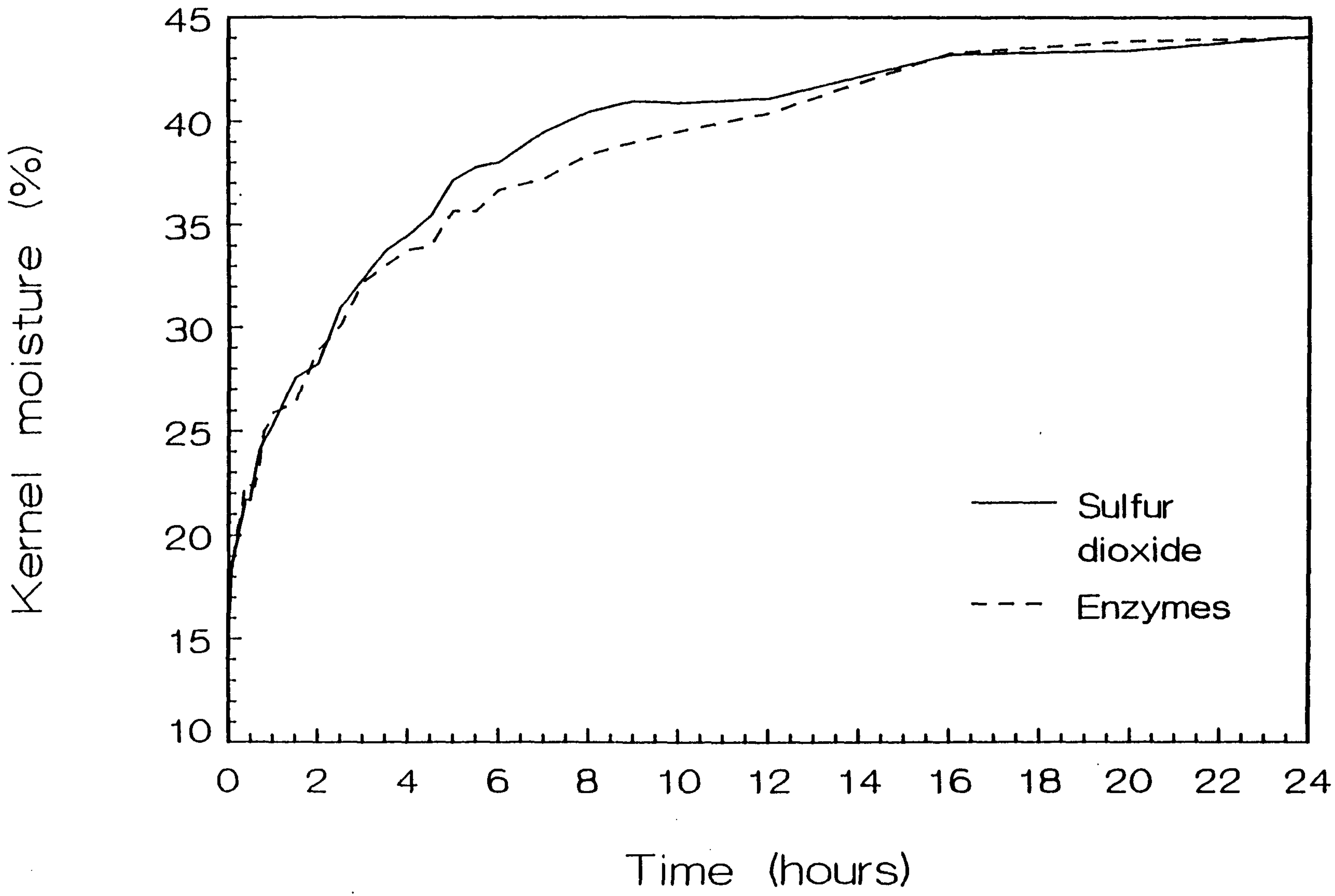
This experiment was performed to determine whether the addition of enzymes increased the rate of water penetration into the corn kernel. Although earlier evidence implicated the enzymes in aiding the penetration of SO_2 and the release of additional solid material, no difference in water absorption was found. The diffusivity constants were calculated to be 4.913×10^{-3} cm^2/hr for the enzyme treated corn versus 4.914×10^{-3} cm^2/hr for the corn treated with SO_2 only. The moisture content of the corn as a function of time is displayed in Figure 7.

Microscopic Examination of Steeped Kernels

Earlier in the batch steeping results section, attention was drawn to the differences in appearance of the corn steeped in enzyme solution and the corn steeped in SO_2 only. The difference is shown in Figure 8.

The grayish-black appearance of corn steeped in enzyme was believed to be caused by enzyme hydrolysis of one or more of the fibrous layers creating a void between the external layers of the kernel. Visual indication of this void would provide tangible support for the evidence observed earlier as a consequence of enzyme steeping. Therefore, cross sections of

Figure 7. Water absorption of corn as a function of time in enzyme and 0.2% sulfur dioxide solution



Kernel moisture (%)
45
40
35
30
25
20
15
10
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)
Sulfur dioxide
Enzymes

Figure 8. Steeped corn kernels from 48 hr (A), 24 hr (B), and enzyme (C) treatments

A

B

C

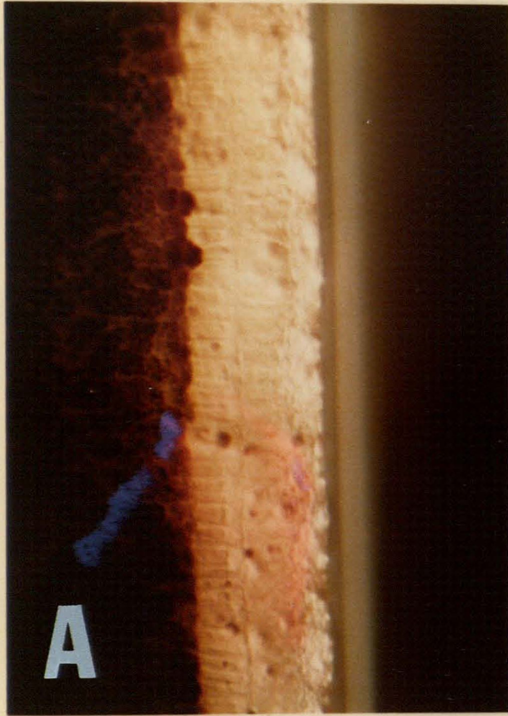
several kernels from each treatment were examined for peculiarities. Clean cross sections without mechanically disrupting the pericarp, however, were very difficult to produce. Evidence of the void in the pericarp is shown in the micrographs in Figures 9 and 10.

The void created in the pericarp of the kernel shown in the micrographs of enzyme-treated corn was found in the vicinity of the cross cell layer (see Figure 1) which is one of the cellulolysic layers most susceptible to carbohydrases. The void was found in quite a few enzyme steeped kernels while most of the 48 hr and 24 hr steeped kernels produced cross sections with intact pericarp similar to those shown in Figures 9 and 10. However, the evidence was not quantified due to the difficulty in producing acceptable cross sections and because of uncertainty in distinguishing between enzymatically separated pericarp and pericarp mechanically separated during sectioning in some of the sections. Therefore, this experimental method did not provide enough tangible evidence necessary to link the carbohydrases to the hydrolysis of the pericarp.

Separation of Fibrous Layers

This exercise was another attempt to identify the mode of action of the carbohydrases in the enzyme mixture by removing

Figure 9. Cross section micrographs of 48 hr (A), 24 hr (B), and enzyme (C) steeped kernels magnified 8.75 times


A

B

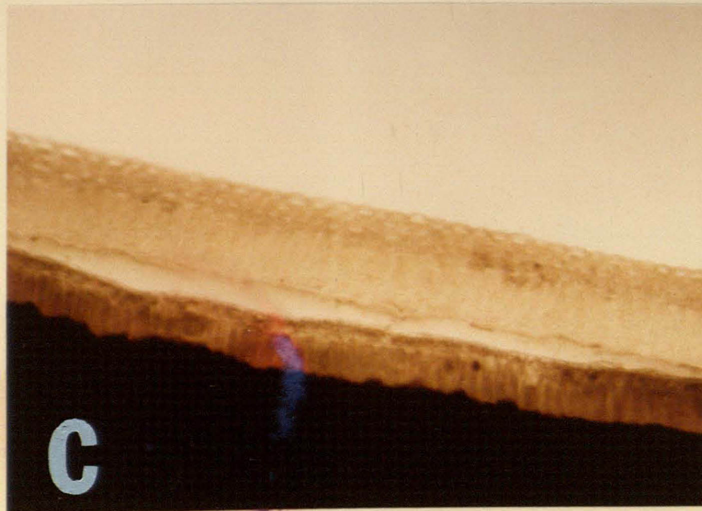
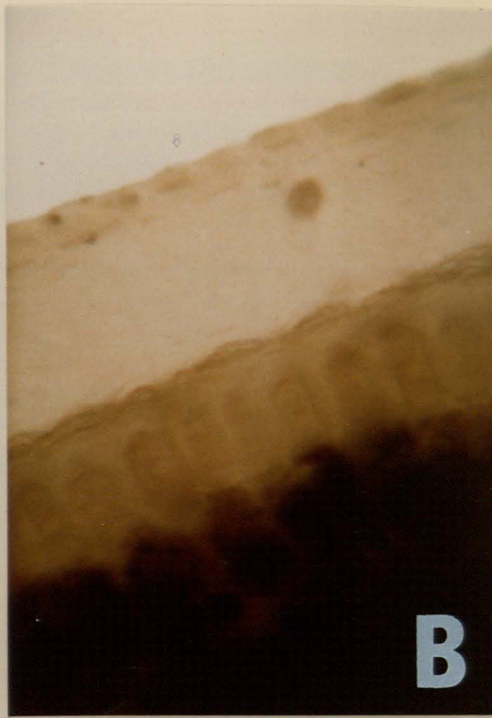

C

Figure 10. Cross section micrographs of 48 hr (A), 24 hr (B), and enzyme (C) steeped kernels magnified 25 times

A


B

C

as many of the external layers from the kernel as possible with a pinch and a pull of the fingers. Although this method may not be very objective, it provided some insight into the possible role of the enzymes.

Of the fifty kernels tested from each treatment, 60% of the enzyme steeped kernels, 10% of the 48 hr steeped kernels, and none of the 24 hr steeped kernels were categorized as detached. A detached sample was one in which the pericarp alone was removed and transparent to light. An attached sample was one in which the seed coat and aleurone layers were removed along with the pericarp and was opaque to light. The results are shown in Figure 11.

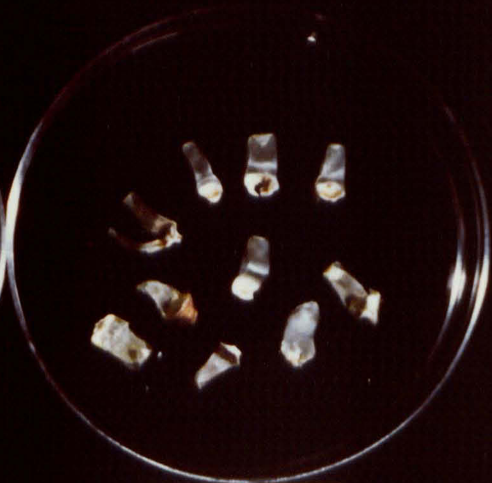
Consequences of Enzyme Addition on Protein/Starch Separation

Most notable consequences of enzyme addition were in significantly reduced levels of protein in the fiber and starch fractions and in significantly increased level of protein in the gluten fraction (enzyme treatment vs 48 hr and 24 hr treatments) which was attributed to the digesting and solubilizing action of bromelain on the matrix proteins. Whether the bromelain hydrolyzed the proteins by penetrating the kernel during steeping or later during wet milling was not investigated. Some enzyme hydrolysis after steeping was possible since all of the enzymes, including bromelain,

Figure 11. Fiber strips separated from 48 hr (A), 24 hr (B), and enzyme (C) steeped kernels

A

B


C

maintain 10 - 40% of their maximum activity at room temperature according to the manufacturer's specifications.

Another notable consequence was the significant increase in starch yield over the 24 hr steeped corn due to action of the carbohydrases and the protease. There was evidence to suggest the bromelain effectively digested the protein matrix releasing more starch from the enzyme steeped corn. Again, whether the protein digestion occurred during or after steeping was not determined.

In promotional literature from the enzyme manufacturer mentioned previously, cellulase was added to an industrial wet milling process at the first grind mill after steeping. They theorized that the cellulase digested the aleurone layer releasing starch normally lost in fiber fraction. An increase in the starch yield of 0.5% was claimed. Possibly the carbohydrases in this study performed in a similar manner during the steeping and/or wet milling process.

CONCLUSIONS AND RECOMMENDATIONS

This study has resulted in the development of a laboratory corn wet milling procedure that produced fractionation results which approached typical commercial wet milling results and met or exceeded previously published laboratory wet milling results for fraction yield and purity. Simulation of commercial steeping was found to be difficult because of the small scale of the laboratory equipment used. However, the steeped corn produced by the countercurrent system developed for this study exhibited excellent wet milling characteristics.

Proof that addition of a mixed enzyme preparation into the steeping phase of a laboratory corn wet milling process to reduce the steeping time and enhance starch/protein separation was established. Consequences, such as significantly superior fraction yields and purities, facilitation of SO_2 penetration, and additional release of solid material support this conclusion. Tangible evidence for the mechanism by which these enzymes effected their action, however, was not completely resolved in this study. Water diffusivity was not improved by employing enzymes. Bran appears to have separated more easily and completely in the presence of the enzymes used in this study.

Starch quality was significantly affected when the steeping period was shortened to 24 hrs due to incomplete

breakdown of the protein matrix. Addition of a mixed enzyme system to the shortened steeping period elevated the starch quality to the level of that produced from the 48 hr steeping period. Also, no detrimental effects on the starch quality were observed from the use of fresh steeping solution with a pH as low as 2.2 or from the addition of the mixed enzyme system. Further work is necessary to gather more information on the feasibility of enzyme addition and the mechanism by which the enzymes affect wet milling performance. Therefore, it is recommended that 1) the mode of action of the enzymes be further studied by labeling or marking the enzymes to trace their path during steeping; 2) the parameters such as temperature, pH, enzyme concentration, and time be altered to determine the most ideal conditions of steeping with enzymes; 3) each enzyme in the mixture be screened for efficacy; 4) the minimum amount of enzymes for steeping corn in a shortened steeping period be determined; 5) enzymes from other sources or manufacturers be investigated for possible substitution into the mixed enzyme system if found to have superior properties; 6) the activity of the enzymes over time be investigated; and 7) the effect of SO_2 on the enzymes be determined.

Some improvements in the laboratory countercurrent steeping system are also possible. Installation of an automated system for the outlet lines from each steeping vessel similar to the system that controls the fresh steep solution

feed would provide for more convenient collection of steep liquor. Replacement of the fresh steep solution prepared from distilled water with a steep solution prepared from commercial processing water may help stabilize the pH in the system.

The information obtained from this investigation, coupled with further research, can be used by the corn wet milling industry to increase corn starch production to satisfy the growing demand for corn starch and reduce processing costs. Enzyme enhancement of starch separation may play a role in further expansion of corn utilization.

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APPENDIX

Table 13. Individual observations for yield and protein composition of mill fractions from batch steeped corn

Fraction	Steeping treatment	Yield (%)	Protein (%)
Germ	48 hr control	7.13	17.68
		5.99	17.26
		7.01	17.37
		6.61	17.84
		6.30	17.72
		6.66	17.91
	24 hr control	7.46	18.69
		6.97	18.21
		7.39	17.86
		7.42	18.44
		7.36	18.46
		7.27	19.15
	Mixed enzyme system	6.70	17.61
		6.34	17.93
		6.86	16.83
		6.66	17.41
		6.82	16.99
		6.63	17.48
	Exp. enzyme product	6.95	17.54
		7.10	17.36
		6.52	19.21
		6.52	19.20
		6.49	19.33
		6.65	18.76
Total fiber	48 hr control	22.23	10.60
		20.69	10.67
		18.32	12.73
		17.69	11.89
		18.82	11.81
		17.44	12.17

Table 13. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Total fiber	24 hr control	28.24	11.59
		25.72	13.38
		25.58	13.24
		24.73	13.57
		24.87	13.55
		25.30	13.19
	Mixed enzyme system	17.86	11.77
		17.76	11.67
		17.11	12.30
		17.93	11.60
		17.56	11.77
		17.56	11.94
	Exp. enzyme product	20.46	12.47
		23.55	12.43
		19.59	12.77
		18.57	12.95
		19.33	13.05
		19.24	12.97
Starch	48 hr control	57.40	0.60
		58.15	0.51
		59.19	0.53
		58.63	0.64
		58.15	0.54
		59.13	0.54
	24 hr control	49.79	1.13
		49.45	1.17
		48.37	1.19
		48.20	1.24
		49.03	1.17
		47.56	1.25
	Mixed enzyme system	57.20	0.60
		58.06	0.52
		57.51	0.48
		57.32	0.53
		57.42	0.50
		57.62	0.50

Table 13. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Starch	Exp. enzyme product	54.60	0.44
		53.52	0.79
		55.55	0.57
		55.05	0.70
		55.41	0.61
		55.10	0.64
Gluten	48 hr control	4.96	54.93
		5.59	51.36
		5.26	53.70
		4.95	60.08
		4.75	62.21
		5.44	53.79
	24 hr control	5.55	43.58
		5.50	44.03
		5.62	42.70
		6.42	40.00
		6.00	43.01
		6.42	43.22
	Mixed enzyme system	5.98	55.65
		5.87	57.66
		6.01	57.26
		5.75	58.70
		5.79	56.96
		5.69	58.24
	Exp. enzyme product	5.03	60.40
		5.19	56.47
		5.44	53.41
		5.68	56.90
		5.61	56.80
		5.51	56.81

Table 13. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Inseparables	48 hr control	3.69	8.09
		3.70	7.63
		3.13	8.46
		4.14	6.93
		4.26	6.79
		3.44	7.17
	24 hr control	7.60	8.38
		6.76	11.10
		6.91	10.93
		7.17	10.28
		6.58	11.79
		7.39	9.18
	Mixed enzyme system	4.83	5.28
		4.57	5.81
		4.52	6.13
		4.31	6.80
		4.44	6.19
		4.41	6.44
	Exp. enzyme product	5.38	8.88
		3.13	10.40
		5.23	9.44
		6.68	6.29
		5.67	8.46
		5.90	8.42
Solubles[a]	48 hr control	5.75	...
		5.88	...
		7.08	...
		7.78	...
		7.72	...
		7.90	...

[a]Protein analysis was not performed on the solubles fraction samples.

Table 13. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Solubles	24 hr control	5.26	...
		5.61	...
		6.13	...
		6.05	...
		6.14	...
		6.07	...
	Mixed enzyme product	7.46	...
		7.41	...
		8.00	...
		8.03	...
		7.98	...
		8.10	...
	Exp. enzyme product	7.50	...
		7.51	...
		7.67	...
		7.49	...
		7.49	...
		7.61	...

Table 14. Individual observations for yield and protein composition of mill fractions from countercurrent steeped corn

Fraction	Steeping treatment	Yield (%)	Protein (%)
Germ	48 hr control	6.88	14.86
		6.69	14.46
		6.67	14.36
		6.66	14.16
		6.77	14.85
		6.63	15.02
	24 hr control	7.00	17.26
		7.28	16.19
		7.06	16.65
		7.21	17.69
		7.20	17.19
		6.95	17.98
	Enzyme	6.65	18.40
		6.78	20.20
		6.84	19.19
		6.67	19.70
		6.68	19.06
		6.70	18.48
Total fiber	48 hr control	10.14	10.11
		10.53	9.90
		10.36	9.73
		10.36	10.06
		10.41	9.74
		10.36	9.88
	24 hr control	11.22	11.21
		11.15	11.41
		11.30	11.30
		12.13	10.78
		11.11	11.07
		12.07	10.74

Table 14. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Total fiber	Enzyme	13.45	8.26
		13.07	7.78
		13.44	8.01
		13.06	7.49
		13.34	7.80
		13.46	8.23
Starch	48 hr control	65.02	0.41
		64.81	0.42
		65.43	0.41
		64.83	0.58
		65.26	0.36
		64.29	0.35
	24 hr control	56.39	0.83
		57.01	0.92
		56.28	0.89
		57.09	0.71
		58.97	0.84
		55.94	0.74
	Enzyme	63.94	0.24
		64.87	0.31
		63.64	0.34
		64.01	0.40
		63.21	0.34
		64.22	0.33
Gluten	48 hr control	5.44	48.40
		5.62	49.18
		5.69	46.81
		5.52	48.16
		5.61	46.65
		5.56	46.25

Table 14. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Gluten	24 hr control	5.98	45.52
		6.29	42.30
		6.44	45.00
		5.95	45.70
		6.66	44.86
		6.69	43.28
	Enzyme	6.60	50.65
		6.62	49.35
		6.65	48.85
		6.68	48.62
		6.64	50.41
		6.71	52.48
Inseparables	48 hr control	4.70	2.52
		4.51	4.31
		4.55	3.59
		4.46	3.47
		4.36	2.86
		4.06	2.76
	24 hr control	11.78	1.86
		10.91	1.21
		11.68	2.40
		10.14	1.87
		8.98	1.68
		11.21	1.90
	Enzyme	2.36	4.08
		2.38	4.44
		2.60	4.44
		1.91	4.73
		2.39	4.56
		2.48	4.97

Table 14. (Continued)

Fraction	Steeping treatment	Yield (%)	Protein (%)
Solubles[a]	48 hr control	7.31	...
		7.84	...
		7.29	...
		8.17	...
		7.60	...
		9.11	...
	24 hr control	7.63	...
		7.35	...
		7.24	...
		7.57	...
		7.08	...
		7.14	...
	Enzyme	7.00	...
		6.28	...
		6.83	...
		7.67	...
		7.74	...
		6.43	...

[a]Protein analysis was not performed on the solubles fraction samples.