

# Enzymatic Milling Product Yield Comparison with Reduced Levels of Bromelain and Varying Levels of Sulfur Dioxide

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## ABSTRACT

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Enzymatic milling (E-Milling) is a process that could potentially replace the sulfur dioxide procedure currently used in all commercial wet-milling facilities. E-Milling incorporates the use of a short water soaking step ( $\leq 6$  hr), a coarse grind, and the use of a protease to release the starch granules from the corn endosperm. E-Milling does not require sulfur dioxide to obtain starch yields equivalent to conventional wet milling; however, the important antimicrobial effects of sulfur dioxide are not duplicated by the enzymatic process. The use of low levels of sulfur dioxide (sufficient for antimicrobial activity) is being proposed as an easily implemented means of microbial control during E-Milling. To assess the effectiveness of E-Milling under these conditions, fraction yields for milling experiments adding sulfur dioxide with and without added enzyme were compared with fraction yields from conventional 24-hr

steeping with 2,000 ppm  $\text{SO}_2$  and 0.55% lactic acid. Because adding enzyme and  $\text{SO}_2$  can both improve product yields and compositions independently, it was necessary to use a reduced level of enzyme (much less than necessary to generate “product quality” material) to observe differences in terms of product yields. The results show significant differences in starch, fiber, total gluten, and insoluble gluten recoveries between samples milled with  $\text{SO}_2$  and enzyme compared with those at the same  $\text{SO}_2$  level without enzyme addition. No significant differences were observed for soakwater or germ yields regardless of the  $\text{SO}_2$  level used. The yield benefits from adding both enzyme and  $\text{SO}_2$  are clearly shown over the addition of each individually, for all coproduct yields with the exception of the yields for germ.

An effective enzymatic process that significantly reduced steeping time in a corn wet-milling process was reported by Johnston and Singh (2001). This was also the first report to demonstrate that the use of a protease alone, without other enzymes or sulfur dioxide addition, was sufficient to reach starch yields equivalent to or exceeding that of the conventional sulfite wet-milling process (Johnston and Singh 2001; Johnston et al 2003). Other enzyme-steeping processes have been developed that nominally decreased the required steeping time or to some extent improved starch recoveries (Hassanean and Abdel-Wahed 1986; Caransa et al 1988; Steinke and Johnson 1991; Steinke et al 1991; Moheno-Perez et al 1999; Serna-Saldivar et al 2003). However, in these studies, enzymes were used in combination with high levels of  $\text{SO}_2$  and no specific class of enzyme or enzymes responsible for improvements were identified.

Using reproducible laboratory fractionation procedures for conventional wet milling (Eckhoff et al 1993, 1996; Dowd 2003), the starch yields from adequately steeped corn are very close to theoretical yields. This makes the differentiation of conventional yields and the potential improvements from an added enzymatic effect difficult, if not impossible, to distinguish statistically, even with the most reliable and reproducible methods. These hindrances, in combination with the diffusion limitations of using intact kernels, effectively prevented researchers from seeing the true potential for enzymes in grain fractionation processes.

In conventional wet milling, sulfur dioxide plays a dual role in processing: the chemical processing agent and the microbial control agent. As the chemical processing agent, sulfur dioxide acts as a reducing agent that disrupts disulfide bond linkages in the protein matrix of the kernel (particularly in the endosperm). This disruption greatly improves the subsequent separation of the kernel com-

ponents using physical separation techniques (size and density separations). As a microbial control agent, it has the effect of eliminating the growth of many microorganisms and, in particular, prohibits the growth of most food pathogens; however, the effectiveness is highly pH dependent. Lactic acid bacteria can grow under moderately high concentrations of sulfites, but these are typically not problem organisms. Many microorganisms are effectively controlled by relatively low levels of sulfite addition with typical food preservation levels of 200–300 ppm (Chichester and Tanner 1972), which are significantly lower than the 1,500–2,000 ppm levels used in the commercial wet-milling processes.

Sulfur dioxide, by disrupting disulfide bond linkages in proteins, can quickly inactivate many enzymes when used at low levels. This is particularly true at typical processing levels (1,500–2,000 ppm). However, there are certain classes of enzymes that can actually be activated by sulfur dioxide (or other reducing agents) and others that retain activity at relatively high sulfite levels. Cysteine proteases, including bromelain, are in this category. Bromelain is an enzyme preparation extracted from the leaves of the pineapple plant and contains at least four distinct cysteine proteases.

Enzymatic milling (E-Milling) is a process that incorporates a short soaking step (4–6 hr) followed by a coarse grind and incubation under controlled conditions with a protease to release the starch-protein interactions before traditional fractionation (Johnston and Singh 2001). E-Milling does not require sulfur dioxide for effective product separation and has the potential to replace the sulfur dioxide procedure currently used in commercial wet-milling facilities. To be used in a continuous commercial operation, an effective microbial control agent would be required in the process. The use of low levels of sulfur dioxide (sufficient for antimicrobial activity) is being proposed as an easily implemented procedure for microbial control during E-Milling while still maintaining the beneficial aspects of the process.

The work presented here was intended to assess the effects of E-Milling under conditions where low levels of sulfur dioxide are present. The objective was not to measure microbial inhibition but rather to use  $\text{SO}_2$  levels adequate for microbial inhibition and determine changes (if any) in product yields as a result of this addition. Initial work with E-milling was done using bromelain; however, another commercial enzyme (GC 106, Genencor International) performs comparably to or better than bromelain enzyme. Because both sulfur dioxide and protease addition contribute to product yield and separation, experimental conditions and controls were

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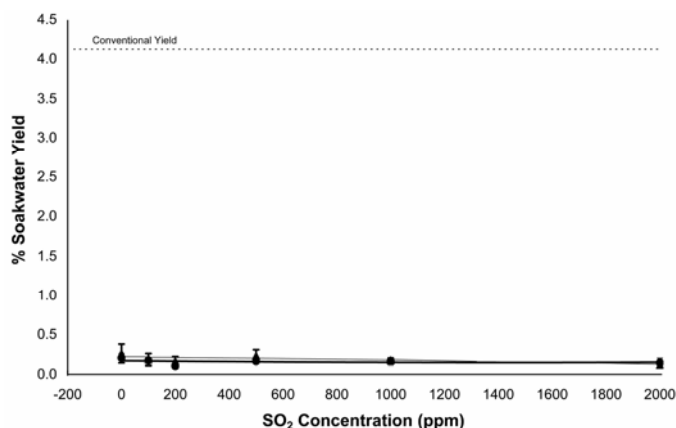
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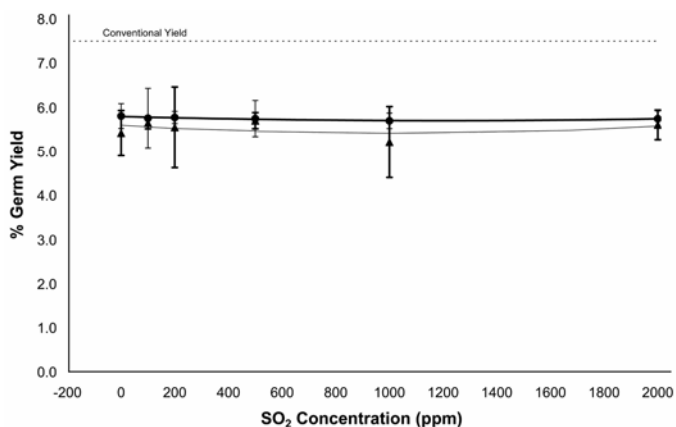
designed so that the beneficial effects of incorporation of each component individually and together could be measured by changes in product yields. As a result, the conditions used were not adequate to produce product quality materials, thus compositional comparisons of the coproducts produced were not useful. The determination of the optimal use of both components in the process was not a goal of the research; however, it should be possible using a similar approach.

## MATERIALS AND METHODS

A yellow dent corn hybrid (Pioneer hybrid 33A14) was grown at the University of Illinois experimental station during the 2002 growing season and was used for all experiments. The corn was field-dried, combine-harvested, and hand-cleaned to remove broken kernels and foreign materials. The corn was weighed and stored in 1-kg bags (wet weight with  $\approx 14\%$  moisture content) at  $4^\circ\text{C}$  until



**Fig. 1.** Soakwater yield comparison between  $\text{SO}_2$  levels incubated without ( $\blacktriangle$ ) and with ( $\bullet$ ) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for steepwater using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of  $\text{SO}_2$  and 0.55% (w/v) lactic acid with a 24 hr steeping time at  $52^\circ\text{C}$ . Error bars show  $\pm 1$  SD of results from duplicate milling experiments done at the indicated  $\text{SO}_2$  levels. Heavy trendline is for experiments with bromelain and  $\text{SO}_2$  added; thin trendline is for  $\text{SO}_2$  addition only.



**Fig. 2.** Germ yield comparison between  $\text{SO}_2$  levels incubated without ( $\blacktriangle$ ) and with ( $\bullet$ ) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for germ using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of  $\text{SO}_2$  and 0.55% (w/v) lactic acid with a 24 hr steeping time at  $52^\circ\text{C}$ . Error bars show  $\pm 1$  SD of results from duplicate milling experiments done at the indicated  $\text{SO}_2$  levels. Heavy trendline is for experiments with bromelain and  $\text{SO}_2$  added; thin trendline is for  $\text{SO}_2$  addition only.

use. Kernel moisture content was measured using a  $103^\circ\text{C}$  convection oven according to Approved Method 44-15A (AACC International 2000). Bromelain was purchased from Sigma-Aldrich Co. All other chemicals were reagent-grade or better.

## Milling Procedures

Conventional milling controls were done by steeping 1 kg of corn in 2,000 ppm  $\text{SO}_2$  at  $52^\circ\text{C}$  with 0.55% lactic acid for 24 hr before grinding. The first grind was done in a Waring blender using a two-prong blunt-edge blade. The blender unit had an adjustable speed controller and a tachometer attached to measure actual motor speed during milling. Grinding conditions were set using actual motor speed to give more reproducible results. Grinding conditions were 4,500 rpm for 3 min for the conventional milling control samples. The remainder of the milling and separation processing was done according to the wet-milling procedure developed by Eckhoff et al (1993). The conventional yields are indicated in Figs. 1–7 by a dotted line and represent the average results of six separate conventional laboratory corn wet-milling experiments done using the standardized process referenced above.

Enzymatic milling and control experiments (without enzyme addition) were done according to Johnston and Singh (2004) by first soaking 1 kg of cleaned corn in 2L of water at  $55^\circ\text{C}$  for 6 hr. After the soaking step, the water (soakwater) was drained and the remaining volume and dry solids content were determined according to Approved Method 44-18 (AACC International 2000). The corn was transferred into a Waring blender and 1.5L of fresh water was added. The corn was coarsely ground using 2,500 rpm for 5 min, 3,000 rpm for 3 min, and 4,000 rpm for 2 min for all experiments (Johnston and Singh 2004). After the first grind, the slurry was transferred to a stainless steel bucket and placed in a water bath at  $48^\circ\text{C}$  with a mechanical stirring device set at  $\approx 20$  rpm. Sodium metabisulfite was added to the slurry to reach the desired concentration and the slurry was adjusted to pH 5.0 with acetic acid. Bromelain was mixed (0.25 g) into the slurry for enzyme treatments after pH levels were stable ( $\approx 5$  min). The pH was monitored throughout the 4-hr incubation period and additional acetic acid was added if necessary. Typically, a total of 2.0–2.5 mL of acetic acid was needed during the incubation to maintain pH. After incubation, fractions were separated and fraction yields were determined using conventional wet-milling procedures developed by Eckhoff et al (1993). Yields were calculated, based on initial dry weight of corn used, and took into consideration the addition of other components added during processing. The levels of  $\text{SO}_2$  were calculated based on the addition of sodium metabisulfite. The actual concentration in the slurry was not measured and at the lower levels was likely completely consumed during treatment.

## Gluten Filtration

The total gluten fraction was collected and the solids content was determined. A 4L subsample of the total gluten fraction was taken after mixing well ( $\approx 35\%$  of the total gluten fraction). This sample was filtered through a Büchner funnel using Whatman #4 filter paper that had been previously weighed. After filtration, the filter cake and paper were separated, dried, and the moisture content was determined. The dry solids content of the filtrate and the filter cake were determined according to Approved Method 44-15A (AACC International 2000) and the soluble and insoluble gluten values were calculated.

## RESULTS AND DISCUSSION

### Soakwater and Steepwater Yield

The yield of soakwater solids was significantly reduced when compared with steepwater from the conventional 24-hr  $\text{SO}_2$  milling procedure (Fig. 1). Yields were  $\approx 4.0\%$  lower in absolute terms, but on a relative basis they were 95% lower for all treatments. At

this stage of the processing, all enzymatic milling samples had been treated equally and no difference between treatments with and without enzyme or from SO<sub>2</sub> treatments would be expected. The reduction of soakwater when compared with the conventional steepwater is due to the reduced soaking time and the solubilizing effects of the reducing agent (SO<sub>2</sub>) and lactic acid (Dailey 2002). Corn samples soaked in water for 24 hr still yield less soakwater solids than an equivalently steeped sample (data not shown).

### Germ Yield

The yield of germ from the enzymatic milling procedures was reduced when compared with the conventional yields by 1–1.5%, regardless of the level of SO<sub>2</sub> addition or the addition of enzyme (Fig. 2). The lower germ recoveries are believed to be the result of the decreased removal of soluble components from the germ (making the germ slightly more dense, thus causing it to sink). During conventional wet-milling, the long steeping time and the high levels of SO<sub>2</sub> and lactic acid remove a significant amount of material from the kernels, mainly from the germ. The remaining germ is relatively high in oil and thus has a lower overall density. In the modified milling process being used here, the soaking time is much shorter and does not have SO<sub>2</sub> or lactic acid present during soaking. This results in the significant differences between steepwater and soakwater yields discussed above and shown in Fig. 1. The germ, still containing these components, has a higher overall density and as a result does not float as well. The measured specific gravity values for the slurries before germ floatation were equal or higher than those for conventional milling. The addition of SO<sub>2</sub> during incubation with or without enzyme addition had no significant effect on germ yield recovery. Recent experiments indicate that increasing the specific gravity by slightly altering the water balance can restore germ yields (results not shown). The addition of other chemicals such as lactic acid could also help improve the floatation of the germ and simultaneously decrease the chances for microbial contamination.

### Fiber Yield

Fiber recovery yields were significantly different when compared with conventional milling samples, as well as comparison between treatments with and without enzyme at each SO<sub>2</sub> level tested (Fig. 3). Without SO<sub>2</sub> addition (0 ppm), the fiber yields for experiments with and without enzyme were 12.6 and 18.5%, respectively. These values were both significantly higher than the conventional fiber yield of 8.4%, indicating that these samples had higher levels of starch when compared with the conventional fiber samples. Previous studies have shown that equivalent yields of fiber can be obtained without addition of SO<sub>2</sub>, provided sufficient enzyme and incubation time are given (Johnston and Singh 2001).

### Starch Yield

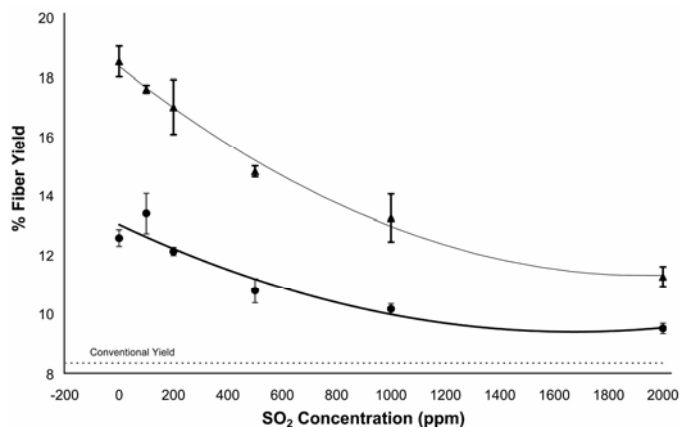
Starch yields were significantly different when compared with starch yields from the conventional wet-milling controls (Fig. 4). They were also significantly different when compared between treatments milled with a particular SO<sub>2</sub> level, with enzyme addition to those milled at the same SO<sub>2</sub> level without enzyme addition. Treatments with both enzyme and SO<sub>2</sub> addition reach conventional starch yields at levels of ≈1,000 ppm SO<sub>2</sub>. The 1,000 ppm SO<sub>2</sub> milling runs with enzyme were not statistically different than the conventional milling yields. Lower levels of SO<sub>2</sub> with enzyme addition resulted in progressively reduced starch yields.

Experiments with SO<sub>2</sub> addition alone during the incubation step resulted in significantly reduced starch recoveries when compared with conventional starch yields, even at the 2,000 ppm level. When directly comparing the effects of SO<sub>2</sub> with and without enzyme addition, it becomes apparent that ≈1,000 ppm of SO<sub>2</sub> addition is required to equal the effects of the enzyme addition alone under these conditions. Additionally, we can also conclude that enzyme activity was not significantly inhibited by the addition of SO<sub>2</sub>. If

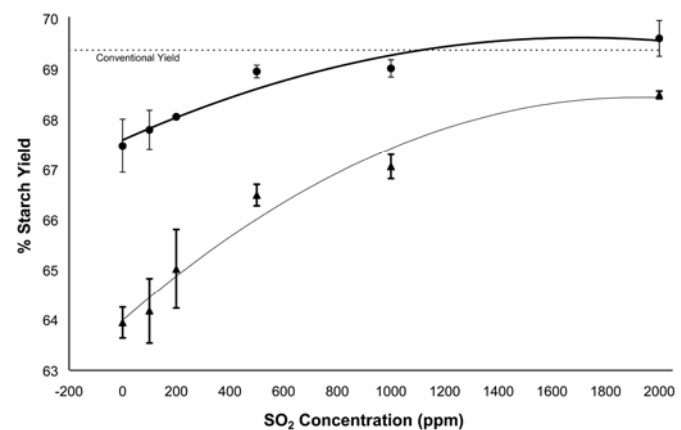
the enzyme activity had been affected, we would have expected the starch recoveries to drop at the higher SO<sub>2</sub> levels to the same yields as with SO<sub>2</sub> addition alone.

### Gluten Yield

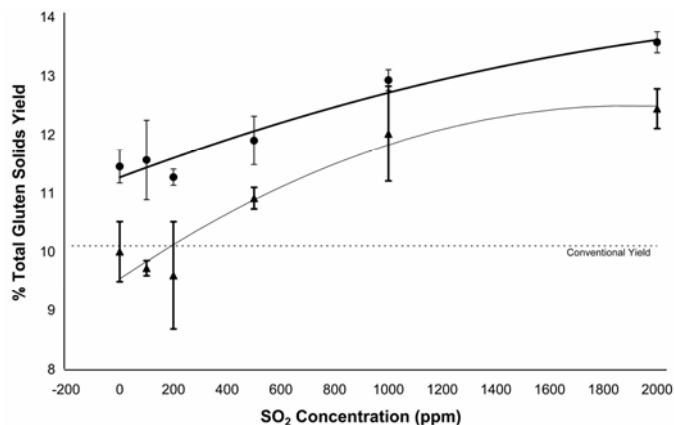
Gluten yields were analyzed as total gluten solids (Fig. 5), as insoluble gluten solids (Fig. 6), and as soluble gluten (Fig. 7). With the exception of the 0, 100, and 200 ppm additions of SO<sub>2</sub> without enzyme, all other treatments were significantly higher than the total gluten solids from the conventional milling runs. This is due to the recovery of soluble components in this fraction, which in the conventional process are recovered in the steepwater fraction. This is confirmed by the significant increase in the soluble gluten fraction for all samples when compared with the conventional control. The recovery of insoluble gluten by filtration of the total gluten samples show significant reductions for the low-level sulfite fractions both with and without enzyme addition.



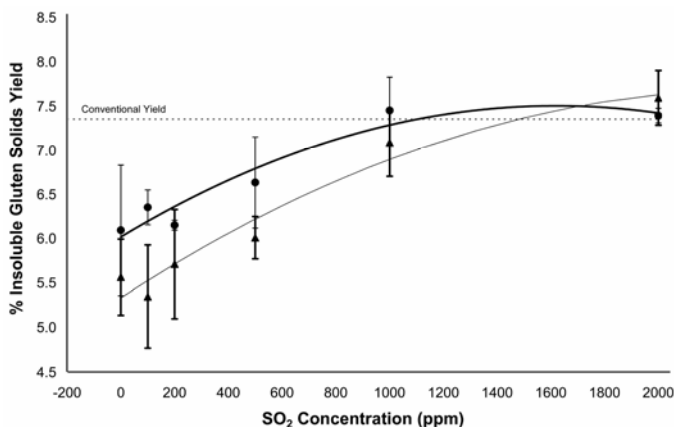
**Fig. 3.** Fiber yield comparison between SO<sub>2</sub> levels incubated without (▲) and with (●) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for fiber using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of SO<sub>2</sub> and 0.55 % (w/v) lactic acid with a 24 hr steeping time at 52°C. Error bars show ± 1 SD of results from duplicate milling experiments done at the indicated SO<sub>2</sub> levels. Heavy trendline is for experiments with bromelain and SO<sub>2</sub> added; thin trendline is for SO<sub>2</sub> addition only.



**Fig. 4.** Starch yield comparison between SO<sub>2</sub> levels incubated without (▲) and with (●) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for fiber using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of SO<sub>2</sub> and 0.55 % (w/v) lactic acid with a 24 hr steeping time at 52°C. Error bars show ± 1 SD of results from duplicate milling experiments done at the indicated SO<sub>2</sub> levels. Heavy trendline is for experiments with bromelain and SO<sub>2</sub> added; thin trendline is for SO<sub>2</sub> addition only.



**Fig. 5.** Gluten solids yield comparison between  $\text{SO}_2$  levels incubated without (▲) and with (●) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for fiber using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of  $\text{SO}_2$  and 0.55 % (w/v) lactic acid with a 24 hr steeping time at 52°C. Error bars show  $\pm 1$  SD of results from duplicate milling experiments done at the indicated  $\text{SO}_2$  levels. Heavy trendline is for experiments with bromelain and  $\text{SO}_2$  added; thin trendline is for  $\text{SO}_2$  addition only.

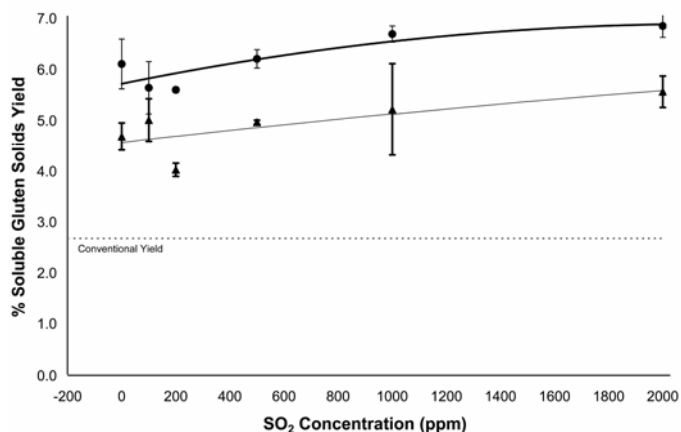


**Fig. 6.** Insoluble gluten (gluten meal) yield comparison between  $\text{SO}_2$  levels incubated without (▲) and with (●) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for fiber using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of  $\text{SO}_2$  and 0.55 % (w/v) lactic acid with a 24 hr steeping time at 52°C. Error bars show  $\pm 1$  SD of results from duplicate milling experiments done at the indicated  $\text{SO}_2$  levels. Heavy trendline is for experiments with bromelain and  $\text{SO}_2$  added; thin trendline is for  $\text{SO}_2$  addition only.

With increased concentrations of  $\text{SO}_2$  during the incubation step, the levels of insoluble gluten increased as well. This increase is likely the result of aggregation of partially denatured protein caused by the  $\text{SO}_2$ -protein interactions. At the 1,000 and 2,000 ppm levels, there were no significant differences between the milling fractions, with or without enzyme addition, and the conventional milling samples.

## CONCLUSIONS

There is clearly a demonstrated complementary effect developed from the addition of  $\text{SO}_2$  and protease in improving wet-milling fraction yields using the E-Milling process. The addition of  $\text{SO}_2$  present in the enzyme incubation step does not appear to inhibit the protease activity and can be included at levels known to be sufficient to control microbial growth during wet-milling. Soak-



**Fig. 7.** Soluble gluten (gluten filtrate) solids yield comparison between  $\text{SO}_2$  levels incubated without (▲) and with (●) 25% bromelain addition. Dotted line represents average conventional corn wet-milling yield for fiber using standard, highly reproducible, laboratory procedure. Value is the average of six individual milling experiments using 2,000 ppm of  $\text{SO}_2$  and 0.55 % (w/v) lactic acid with a 24 hr steeping time at 52°C. Error bars show  $\pm 1$  SD of results from duplicate milling experiments done at the indicated  $\text{SO}_2$  levels. Heavy trendline is for experiments with bromelain and  $\text{SO}_2$  added; thin trendline is for  $\text{SO}_2$  addition only.

water and germ yields were not affected by the addition of enzyme alone or enzyme with  $\text{SO}_2$  addition as high as 2,000 ppm. Starch, fiber, total gluten, and insoluble gluten yields were all improved with the addition of enzyme and  $\text{SO}_2$  over the use of either alone. Utilizing these results, it should be possible to determine the optimal balance between  $\text{SO}_2$  addition and enzyme addition to minimize the overall operation costs.

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