New formulations of mushroom chitosan fiber as a natural and vegan dual function fining agent and antimicrobial for ciders, seltzers, beer, and wine

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Introduction

As consumers become more health conscious and brewers look for more environmentally friendly production practices, the need for natural fining agents and antimicrobials have become increasingly sought after. Available fining agents and antimicrobials are largely synthetic or animal derived. Chitosan is a natural fiber that possesses a strong cationic charge, ideal for binding haze-causing material, as well as being effective at inhibiting the growth of spoilage causing microorganisms. The cationic charge of chitosan is due to the protonation of amino groups on the fiber's backbone[1,2], allowing it to function as a polyelectrolyte. Chinova Bioworks' researchers investigated how a new source of chitosan fiber, extracted from white button mushroom, impacted the clarification of fermented drinks compared to that of chitosan extracted from the traditional sources of *Aspergillus niger* fungus and crustacean shells. Additionally, the antimicrobial effect of the residual chitosan was determined through challenge testing against several common spoilage species of bacteria, mold, and yeast in alcoholic fined cider and seltzer.

Materials and Methods

Chitosan and Molecular Analysis
White Button mushroom (*Agaricus bisporus*) chitosan (Mycobrio™, Chinova Bioworks, Canada) was compared to chitosan derived from *Aspergillus niger* (QI UP Xc, Institut Œnologique de Champagne, France), and chitosan derived from shellfish (MilliporeSigma, USA). Molecular analysis was done by a 400 MHz proton Nuclear Magnetic Resonance (NMR) Spectrometer, where chitosan samples were dissolved in a mixture of D2O and DCl and analyzed at 60°C.

Turbidity
Measurement of turbidity was done with the Orion AQ3010 Turbidity Meter (Thermo Scientific) according to the manufacturer's protocols. Microbiology plating was performed using standard aseptic plating techniques on Potato Dextrose Agar (PDA) for yeast and molds, and Tryptic Soy Broth (TSB) for bacteria.

Cider and Seltzer Fermentation
Cider was fermented from juice provided by a local cider producer, by addition in a 10L carboy system with a cider specific brewer's yeast combined with yeast nutrient. The primary fermentation was carried out at ambient room temperature (70°F) for 7 days. The initial specific gravity was 1.050. Each of the chitosan samples were added at the start of the secondary fermentation, directly after the cider was transferred off the lees from the primary fermentation. Chitosan was added at 100ppm based on the dry weight of chitosan. QI UP Xc was prepared as per manufacturer instructions, by mixing the powder into 10X its weight in chlorine free water prior to addition. Samples were taken at time 0 (before chitosan addition), and after 24, 48, and 72 hours, and the Nephelometric Turbidity Unit (NTU) recorded. Seltzer was fermented from a standard dextrose base kit at ambient room temperature (70°F) for 10 days, and the chitosan samples were added during secondary fermentation when the specific gravity was 1.018. Chitosan was added at 100ppm based on the dry weight of chitosan. QI UP Xc was prepared as per manufacturer instructions, by mixing the powder into 10X its weight in chlorine free water prior to addition. Samples were taken at time 0 (before chitosan addition), and after 24, 48, and 72 hours, and the Nephelometric Turbidity Unit (NTU) recorded.

Antimicrobial Challenge Test in Cider
The antimicrobial effect of each of the three sources of chitosan was tested using an antimicrobial challenge test similar to that of the United States Pharmacopeia (USP) 51 method. Challenge microorganisms were added to the cider after the completion of 72 hours of fining and after racking. The microorganisms included *Aspergillus niger* (mold), *Zygosaccharomyces bailii* (yeast), and *Lactobacillus brevis* (bacteria). Each microorganism was individually inoculated into the clarified cider at a starting concentration of Log 3 CFU/mL (1000 CFU/mL). Samples were incubated at 86°F and sampled at day 1, 4, 7, 14, and 21 using standard dilution and plating techniques.

Results

Molecular Analysis of Mushroom, Fungal, and Crustacean Chitosan
Chitosan from white button mushroom (*Agaricus bisporus*), fungus (*Aspergillus niger*), and crustacean source were analyzed for their key molecular characteristic, the perfect degree of deacetylation (DDA), which is understood to be critical for the performance of fining as the polysaccharide has more free cationically charged amino groups the more it is deacetylated[3].The percent DDA was measured by proton NMR as per the method of Lavertu et al., 2003[4] where 10mg chitosan samples are dissolved in a 2mL solution of 20% deuterium chloride in deuterium oxide and left for 30minutes. The % DDA is calculated by Lavertu's equation using the integrals of the characteristic chitosan peaks. Based on the spectra obtained, it can be calculated that the various chitosans have a degree of deacetylation of 73% (crustacean), 85% (fungal), to the highest being 94% (white button mushroom), as shown in *Table 1*.

Table 1 – Percent degree of deacetylation of various chitosan sources

Brand Name	Supplier	Source	DDA (%)
Mycobrio™	Chinova Bioworks	White Button Mushroom (*Agaricus bisporus*)	94%
QI UP Xc	IOC	*Fungus (Aspergillus niger)*	85%
N/A	MilliporeSigma	Crustacean	73%

Fining Ability of Mushroom, Fungal, and Crustacean Chitosan
Each of the chitosan samples exhibited significant fining ability of both the alcoholic cider and the alcoholic seltzer. The alcoholic cider started with an initial NTU value of 110, and without any fining agent naturally settled to a turbidity of 59 NTU's over the course of 72 hours. The fungal chitosan had the slowest fining ability of the three chitosans at the 12 hour point, only 12 NTU's lower than the control (95 NTU's), whereas the crustacean chitosan was less than half of the control at 48 NTU's. The White Button Mushroom chitosan was the most rapid at fining the cider, going to 14 NTU's at the 12 hour mark. At the end of 72 hours the crustacean chitosan ended up being the worst performing, only reaching 13 NTU's, where the fungal derived chitosan ended at 7 NTU's, and the white button mushroom chitosan at 2 NTU's as shown in *Figure 1*.
The alcoholic seltzer had a much higher turbidity then the alcoholic cider, with a starting NTU value of 543. Over the course of the 72 hours each chitosan showed an improvement over the control in fining, where the fungal chitosan ended up with a final turbidity of 16 NTU's, the crustacean with a final turbidity of 6 NTU's, and the white button mushroom chitosan with the lowest turbidity of just 3 NTU's. The the seltzer's trial there was a significant early difference in the chitosan's performance, the white button mushroom achieved a turbidity reading of 210 NTU's in 12hours, whereas the crustacean and fungal chitosan had 348 and 412 respectively, a minor difference compared to the control which was 414 NTU's, as shown in *Figure 2*.

Figure 1 – Cider fining trial with different chitosan sources

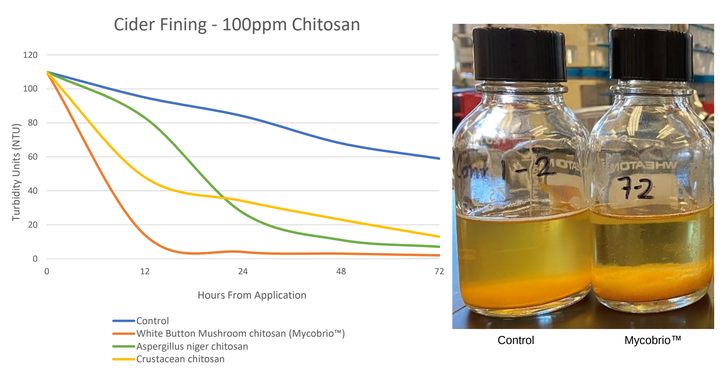
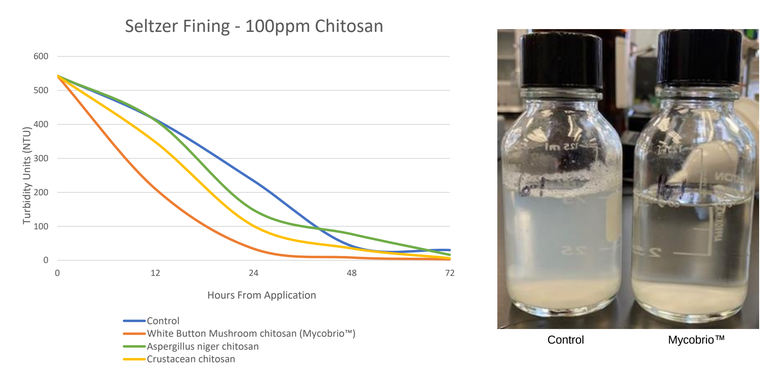
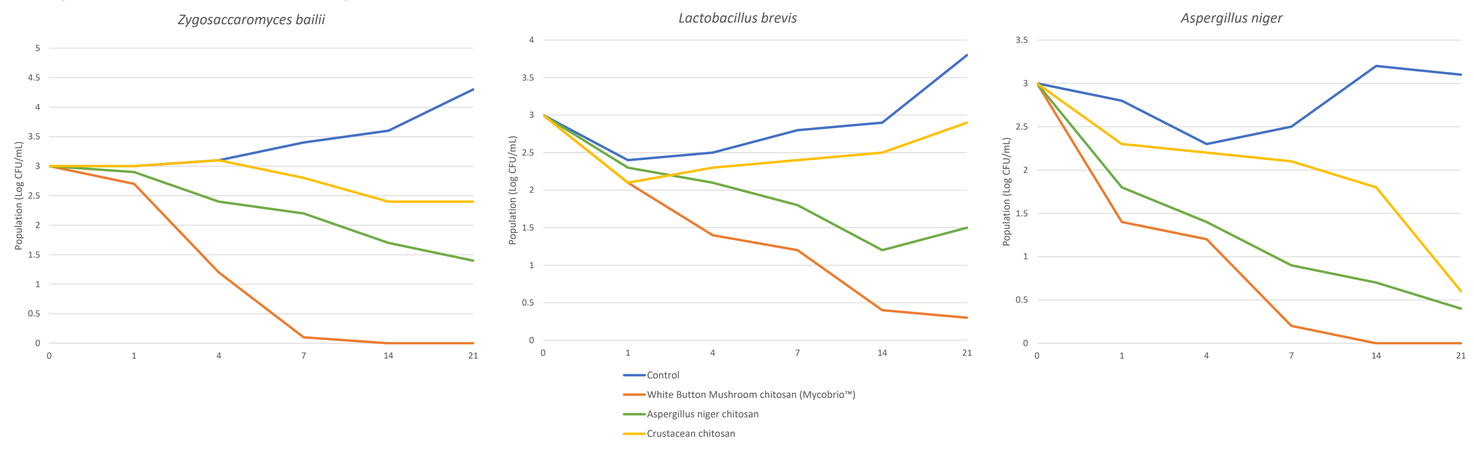


Figure 2 – Seltzer fining trial with different chitosan sources



Antimicrobial Activity of Mushroom, Fungal, and Crustacean Chitosan
The antimicrobial activity of the remaining chitosan in the alcoholic cider was determined by a conventional antimicrobial challenge tests.
The cider was inoculated with Log 3 CFU/mL (1000 CFU/mL) of each of the spoilage organisms; being *Zygosaccharomyces bailii*, *Lactobacillus brevis*, and *Aspergillus niger*. Each sample of chitosan was compared to a control of the cider (no chitosan).
From the initial *Zygosaccharomyces bailii* population of Log 3 CFU/mL, the white button mushroom chitosan exhibited the highest antimicrobial activity. At the 21 day point, the population of *Z. bailii* had been reduced to 0 Log CFU/mL by the white button mushroom chitosan, whereas the fungal *Aspergillus niger* chitosan was only reduced to 1.4 Log CFU/mL and the crustacean chitosan was 2.4 Log CFU/mL. The control grew considerably over this 21 day period, to a final population of 4.3 Log CFU/mL.
From the initial *Lactobacillus brevis* population of Log 3 CFU/mL, the white button mushroom chitosan exhibited the highest antimicrobial activity. At the 21 day point, the population of *L. brevis* had been reduced to 0.3 Log CFU/mL by the white button mushroom chitosan, whereas the fungal *Aspergillus niger* chitosan was only reduced to 1.5 Log CFU/mL and the crustacean chitosan was 2.9 Log CFU/mL. The control grew considerably over this 21 day period, to a final population of 3.8 Log CFU/mL.
From the initial *Aspergillus niger* population of Log 3 CFU/mL, the white button mushroom chitosan again exhibited the highest antimicrobial activity. At the 21 day point, the population of *A. niger* had been reduced to 0 Log CFU/mL by the white button mushroom chitosan, whereas the fungal *Aspergillus niger* chitosan was reduced to 0.4 Log CFU/mL and the crustacean chitosan was 0.6 Log CFU/mL. The control grew considerably over this 21 day period, to a final population of 3.1 Log CFU/mL. All results are shown in *Figure 3*.

Figure 3 – Antimicrobial challenge test results in fined cider



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Conclusion

As more brewer's look to sustainable and environmentally friendly practices, the technology and processes used for fining beers, ciders, seltzers, and wines after fermentation is increasingly important. Practices like cold crashing, centrifuging, or filtration are energy and time consuming, and are an impediment to sustainable brewing. Chitosan has long been investigated for its ability to rapidly fine haze from alcoholic fermented beverages, but chitosan traditionally sourced from crustacean origin is not regarded as a sustainable ingredient and is poor performing compared to synthetic alternatives. The other source of chitosan, from the fungal kingdom, either *Aspergillus niger* fungus or recently the new source of White Button mushrooms (*Agaricus bisporus*) offers a sustainable and green alternative to crustacean derived chitosan. The use of chitosan from White Button mushrooms shows exceptional fining activity when compared to other sources of the natural fiber, as demonstrated by the testing done on cider and seltzers made at pilot scale. Along with an improvement in the fining ability, the White Button mushroom chitosan also displayed a significantly higher residual antimicrobial activity when tested against three common spoilage microorganisms. White Button mushroom chitosan, due to its molecular characteristics, is an improved form of chitosan for brewers looking to rapidly fine their beverage, along with having the added benefit of prolonged shelf life due to its residual antimicrobial activity.