# Flocculation and Haze Removal from Crude Beer Using In-House Produced Laccase from *Trametes versicolor* Cultured on Brewer's Spent Grain

Gurpreet Singh Dhillon,[†] Surinder Kaur,[†,‡] Satinder Kaur Brar,*[,†] and Mausam Verma[§]

[†]INRS-ETE, Université du Québec, 490 Rue de la Couronne, Québec, G1K 9A9 Canada

[‡]Department of Mycology & Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University (BHU), Varanasi-221005, India

[§]Institut de recherche et de développement en agroenvironnement inc. (IRDA), 2700 rue Einstein, Québec (Québec), G1P 3W8 Canada

**ABSTRACT:** The potential of brewer's spent grain (BSG), a common waste from the brewing industry, as a support-substrate for laccase production by the well-known laccase producer *Trametes versicolor* ATCC 20869 under solid-state fermentation conditions was assessed. An attempt was made to improve the laccase production by *T. versicolor* through supplementing the cultures with inducers, such as 2,2-azino bis(3-ethylbenzthiazoline-6-sulfonic acid), copper sulfate, ethanol, gallic acid, veratryl alcohol, and phenol. A higher laccase activity of $13506.2 \pm 138.2$ IU/gds (gram dry substrate) was obtained with a phenol concentration of 10 mg/kg substrate in a tray bioreactor after 12 days of incubation time. The flocculation properties of the laccase treated crude beer samples have been studied by using various parameters, such as viscosity, turbidity, $\zeta$ potential, total polyphenols, and total protein content. The present results indicated that laccase (25 IU/L) showed promising results as a good flocculating agent. The laccase treatment showed better flocculation capacity compared to the industrial flocculation process using stabifix as a flocculant. The laccase treatments (25 IU/L) at $4 \pm 1$ °C and room temperature have shown almost similar flocculation properties without much variability. The study demonstrated the potential of in-house produced laccase using brewer's spent grain for the clarification and flocculation of crude beer as a sustainable alternative to traditional flocculants, such as stabifix and bentonite.

**KEYWORDS:** brewer's spent grain, flocculation, inducers, *Trametes versicolor*

## ■ INTRODUCTION

Laccases (*p*-diphenol/dioxygen oxidoreductases; EC 1.10.3.2) are particularly abundant in white-rot fungi, which are the only organisms able to degrade the whole wood components.[1] In particular, the genus *Trametes* is assumed to be one of the most efficient lignin degraders. Among them, *Trametes versicolor* has been subject of extensive research. An array of industrial applications for laccases has been proposed which include pulp and paper, textile, organic synthesis, environmental, food, pharmaceutical, and nanobiotechnology. A few laccases are at present in the market for textile, food, and other industries, and more candidates are being actively developed for future commercialization. Many laccase substrates, such as unsaturated fatty acids, phenols, and thiol-containing proteins, are important components of various foods and beverages. Their modification by laccase may lead to sustainable processes with new functionality, quality improvement, or cost reduction. Therefore, considering the industrial importance of laccases, future studies to enhance the laccase production ability of microorganisms is necessary.[2]

In the food sector, the brewing sector holds a strategic economic position, with the annual world beer production exceeding 1.8 billion hectolitres in 2008 (www.globalmalt.de). Beer is the fifth most consumed beverage in the world after tea, carbonates, milk, and coffee. During production, beer alternately goes through three chemical and biochemical reactions (mashing, boiling, fermentation, and maturation) and three solid liquid separations (wort separation, wort clarification, and rough beer clarification).[3] Beer is a complex mixture of different chemical compounds, such as ethanol, organic compounds (aroma), salts, and phenolic compounds (color and taste). The storage life of beer depends on different factors, such as haze formation, oxygen content, and temperature.

In fermentation product recovery, the initial step of cell separation from the fermented broth frequently causes the largest drop in the overall extraction efficiency. The process of asexual aggregation of cells also known as flocculation has been the subject of significant biotechnological interest due to its relevance for many industrial fermentation processes. Flocculation aids cell-broth separation by increasing the effective biomass particle size. Flocculation can be defined as the "phenomenon wherein yeast cells adhere in clumps and either sediment rapidly from the medium in which they are suspended or rise to the surface".[4] Flocculation could lead to significant improvements in the processing of biotechnological fermentation products, such as beer, wine, foods, and biofuels, among

others. Besides, the importance of flocculation in natural environments, it also controls phenotypes that have become relevant targets for the improvement of microorganisms in industrial processes. The ability of some microorganisms to display controlled asexual aggregation (also known as bioflocculation), which can lead to extensive and highly compact groups of cells or flocs, has been exploited by fermentation industries to improve various processes, including yeast-based processes, such as brewing, wine making, and bioethanol production, among others.[5−7] Besides the cost saving nature of yeasts (bioflocculation), i.e. self-clearing beers at the end of fermentations, the liquid fermented products can be clarified by other biocatalysts, such as laccases. For cost-effective production of alcoholic beverages, such as beer, inexpensive, sustainable, and environmental friendly clarification methods are sought. The efficiency of primary separation in modern downstream processing operations can be improved by using sustainable and inexpensive biocatalysts.

After cell separation, the most frequently encountered problem of haze formation is due to a cross-linking of polyphenols (tannin) causing protein precipitation and to small quantities of naturally occurring proanthocyanidins. This type of complex is commonly found as a chill-haze and appears during cooling processes but may redissolve at room temperature or above. Even products that are haze-free at the time of packing can develop this type of complex during long-term storage. Thus, the formation of a haze has been a persistent problem in the brewing industry that influences the consumer's perception of the product quality. During brewing, various off-flavor compounds (e.g., trans-2-nonenal) are formed from off-flavor precursors resulting from the reaction of oxygen with fatty acids, amino acids, proteins, and alcohol. Laccases could be added at the end of the process in order to remove the unwanted oxygen in the finished beer, and thereby, the storage life of beer can be enhanced.

Laccases are considered to be environmental friendly and of relatively lower cost. The latter can be further reduced if laccase is produced by means of a solid-state fermentation (SSF) technique (Koji fermentation), especially working with substrates of low cost, such as agro-industrial wastes. In fact, the reutilization of food processing and the agricultural industry wastes offers numerous advantages from both economical and environmental points of view. Furthermore, laccase activity in fungal cultures can be increased by the addition of different aromatic compounds, such as gallic acid, ferulic acid, xylidine, guaiacol, syringaldazine, ethanol, phenol, and veratryl alcohol, which have been widely used to induce laccase production.[8−10]

In the context of economical and sustainable process development, brewer's spent grain (BSG) is a promising substrate for the production of laccase, the target enzyme of the present study, due to the presence of cellulose content and other vital nutrients. *Trametes* constitutively secretes a considerable level of extracellular enzyme laccase in a defined growth medium. Laccase production is constitutive in most white-rot fungi and can be easily enhanced by the addition of different inducers into the culture medium.

In this context, the study was carried out to evaluate the potential of BSG as a solid substrate to cultivate white-rot fungi *Trametes versicolor* for the bioproduction of laccases through SSF. Various inducers, such as 2,2-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), copper sulfate (CS), ethanol (EtOH), gallic acid (GA), veratryl alcohol (VA), and phenol, were supplemented in BSG for enhanced laccase production.

The laccase production abilities of the *T. versicolor* under different culture conditions, such as flasks and plastic trays, were also compared. BSG, a byproduct of brewery fermentation, has not been investigated so far for laccase production via koji fermentation in trays. The in-house produced enzyme using beer industry waste was used for the clarification (haze removal) and flocculation of yeast cells and other proteinaceous matter present in the crude beer samples: (1) beer [simple] and (2) beer [with polyphenols] as a sustainable alternative to traditional flocculant bentonite.

## ■ MATERIALS AND METHODS

**Microorganism Procurement and Maintenance.** The white-rot fungus *Trametes versicolor* ATCC 20869 was selected as a suitable organism for bioprocessing of SSF for its potential for higher enzyme production. The microorganism was grown on potato dextrose agar (PDA) Petri plates and was incubated at $30 \pm 1$ °C for 10−12 days. The culture plates were stored at $4 \pm 1$ °C and subcultured every 4 weeks.

**Substrate Procurement and Pretreatment.** Brewer's spent grain (LA BARBERIE, Quebec, Canada) was employed as solid support and substrate. The proximate composition of BSG is provided in Table 1. The moisture content of BSG was analyzed using a moisture analyzer (HR-83 Halogen, Mettler Toledo, Switzerland).

**Table 1. Proximate Composition of Brewer'S Spent Grain**

| biomass components | composition (DW basis)[a] |
|---|---|
| moisture % (w/w) | $65.4 \pm 1.2$ |
| total carbon (g/kg) | $106.9 \pm 4.8$ |
| cellulose (% DW) | $13.8 \pm 2.4$ |
| hemicellulose (% DW) | $30.0 \pm 4.2$ |
| lignin (% DW) | $12.4 \pm 1.3$ |
| total carbohydrates (% DW) | $34.5-43.3$ |
| protein (% DW) | $23.9 \pm 1.24$ |
| lipid (% DW) | $7.9 \pm 1.4$ |
| ash (%) | $2.6 \pm 0.3$ |
| reducing sugars (% DW) | |
| glucose | $21.5^{b}$ |
| fructose | |
| sucrose | |
| arabinose | $8.5^{b}$ |
| galactose | $1.3^{b}$ |
| xylose | $17.6^{b}$ |

[a]DW= dry weight. [b]% of monosaccharide on dry weight basis.

**Solid-State Laccase Bioproduction in Flasks and Static Tray Bioreactor.** The outline of the experimental plan is given in Figure 1. Brewer's spent grain having a moisture content of 75% (v/w) was used for laccase production. The cultures were performed in cotton-plugged Erlenmeyer flasks (500 mL) containing 40 g of BSG. Tween 80 (0.2% w/w) was added in all flasks to stimulate the secretion of extracellular enzymes. The cultures were supplemented with laccase-inducing compounds at the beginning of cultivation depending on the experiment. Inoculation was carried out directly in the Erlenmeyer flasks, and the medium was sterilized by autoclaving at $121 \pm 1$ °C for 30 min. After cooling, the medium was inoculated with three agar plugs (diameter, 3 mm), from an actively growing fungus on PDA plates per Erlenmeyer flask.

The configuration of plastic trays employed in this work was 40 cm (length), 25 cm (breadth), and 12 cm (height). In brief, 500 g of BSG was separately autoclaved in 2 L beakers and was transferred to sterilized trays, and inoculation was carried out directly in the tray bioreactor with agar plugs (diameter, 3 mm, 3 plugs/40 g BSG) picked up from the upper portion of an actively growing fungus on PDA plates and mixed with the substrate. The flasks and bioreactor were
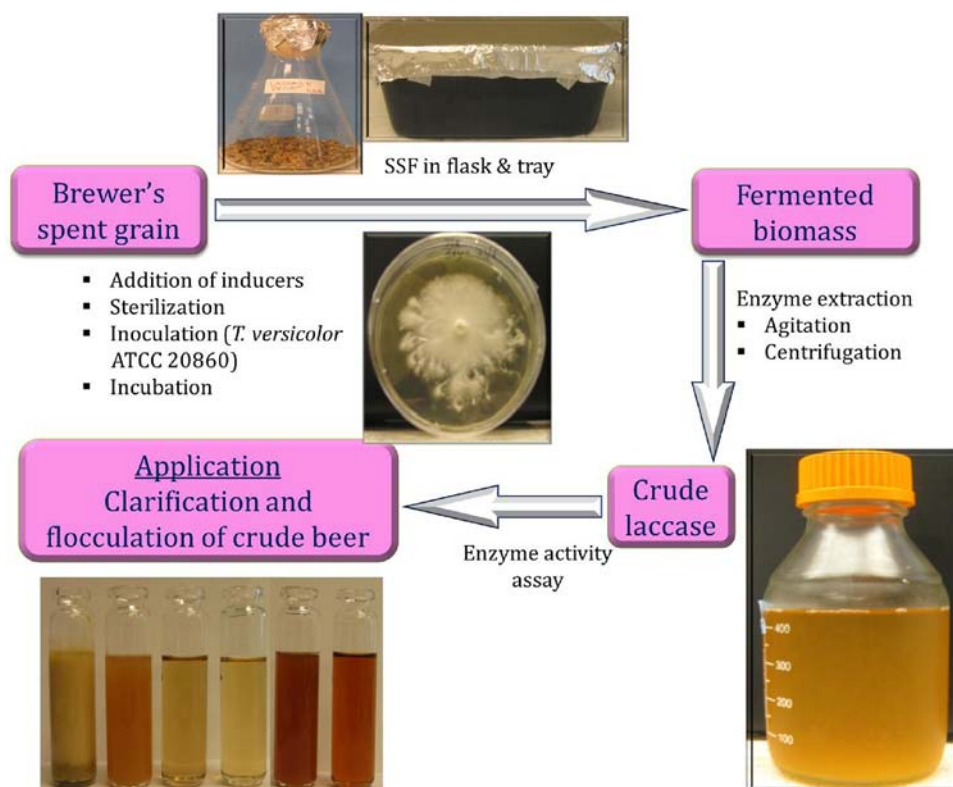
**Figure 1.** Flow chart showing the bioproduction of laccase through SSF and its application for clarification and flocculation of crude beer.

kept in an environmental chamber at 30 ± 1 °C and 90% humidity with passive aeration, in complete darkness under sterilized conditions. Duplicate experiments were run for comparison, and samples were analyzed in triplicate. The values in the figures correspond to mean values with a standard deviation lower than 10%.

**Enzyme Extraction and Assay.** Laccase activities were analyzed at 24-h intervals from extracted samples. About 1.5 g of a wet fermented sample was harvested from each flask after every 24 h under aseptic conditions and dispensed in 15 mL of sodium phosphate buffer (50 mM, pH 6.5, 10:1, v/w). The enzyme extraction was carried out by incubating the samples in a wrist action shaker (Burrell Scientific, Pennsylvania, USA) for 20 min. After incubation, the samples were centrifuged at 9000$g$ and 4 ± 1 °C for 15 min, and supernatant was analyzed for enzyme activities and total protein content. Laccase activity was determined spectrophotometrically with ABTS (2,2′-azino-di[3-ethylbenzo-thiazolin-sulfonate]) as a substrate at 420 nm ($\varepsilon$ = 3.6 × 10$^4$ cm$^{-1}$ M$^{-1}$).[11] One enzyme activity unit was defined as the amount of enzyme that oxidized 1 $\mu$mol of ABTS per min. The laccase activity was expressed in IU/gds (gram dry substrate).

**Fungal Viability Assay.** The viability of fungal mycelium was assessed using the most probable number (MPN) method.[12] The viability assay was used as an indicator or measure of the growth in terms of the amount of living fungal biomass in koji fermentation. In brief, 1 g of fungal-colonized fermented substrate was diluted in an appropriate amount of sterilized distilled water. Afterward, it was homogenized with Ultra-Turrax for 30 s and proper dilutions were made for these samples. In total, five dilutions per sample were used for the viability assay. Each dilution (6 × 10 $\mu$L) was aseptically poured on an agar plate at six different spots, and for each sample, two plates were made. The plates were incubated for 24 h, and the growing spots were calculated. While calculating the results, the first dilution where all the pipetted spots did not grow, the last dilution where at least one pipetted spot shows positive results, and all the samples between these spots were taken into consideration. The results in colony forming units (CFUs) were calculated according to the following formula:

$$MPN = P(N_n \times N_k)0.5 \tag{1}$$

where $P$ = the number of positives in all the accounted series; $N_n$ = amount of sample in negative parallels (g); $N_k$ = amount of sample in all the accounted series (g).

**Applications of Laccases for Flocculation and Clarification of Crude Beer.** The in-house produced laccase (10, 25, 50, 100, and 200 IU/L) was used for the flocculation and clarification of two different crude beer samples: (1) beer [simple] and (2) beer [with polyphenols]. The later beer is rich in polyphenols and is sold under the label biological beer. The final content of phenolic components of beer depends on both the raw materials and the brewing process. For quality control, it is necessary to evaluate phenolic compounds by a rapid analytical method, as they can affect beer flavor and stability. The haze formation occurs in the latter sample due to the presence of polyphenols. The experiment was carried out in 500 mL Erlenmeyer flasks at 4 ± 1 °C and room temperature (RT). Samples were withdrawn at 12 h intervals until a 72 h incubation time, and they were analyzed for viscosity, turbidity, $\zeta$ potential, total polyphenols, and protein content.

The total polyphenol content in the clear upper layer liquor samples settled during flocculation was measured spectrophotometrically by the FC method using gallic acid as standard by the modified method as described by the International Organization for Standardization (2005).[13] The total polyphenols were expressed as gallic acid equivalents (GAE) in milligrams per liter. The soluble protein content in the fermented liquor during flocculation was determined.[14] $\zeta$ potential during flocculation was measured at room temperature using a Zetaphoremeter (version 4.30, CAD Instrumentations, France) and was expressed in millivolts. The turbidity during flocculation was measured using a turbidity meter having an accuracy of ±2 Nephelometric Turbidity Units (NTU) and a tungsten light source (Model 2020e EPA, LaMotte, MD, USA) and was expressed as NTU. The rheological property of the brewery liquor during laccase treatment was determined by using a rotational viscometer Brookfield DV II PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA) equipped with Rheocalc32 software (for rheological models).

7897

dx.doi.org/10.1021/jf301747z | J. Agric. Food Chem. 2012, 60, 7895−7904

Three different spindles, namely, SC-34 (small sample adaptor), L-22, and ULA (ultralow centipoise adapter) were used with a sample cup volume of 20−50 mL (spindle dependent). The gaps between spindle and sample chamber were 1.235 mm for ULA (viscosity range, 1.0−30 mPa·S) and 4.830 mm for SC-34 (viscosity range, $\geq$30 mPa·S) to adapt to the analyzed samples of beer.

**Statistical Analysis.** All the experiments were conducted in duplicates, and data presented are an average of three replicates along with the standard deviation ($\pm$SD). The database was subjected to an analysis of variance (ANOVA), using the Statistical Analysis System Software (STATGRAPHICS Centurion, XV trial version 15.1.02 year 2006, Stat Point, Inc., USA), and the results, which have $p < 0.05$, were considered as significant. Standard errors and error bars presented in the tables and figures, respectively, were calculated using untransformed data in ANOVA.

## ■ RESULTS AND DISCUSSION

Laccase bioproduction was carried out through koji fermentation in flasks and plastic trays using BSG as solid support by *T. versicolor*. BSG is a byproduct of the brewery industry and can be potentially used for laccase production, owing to the presence of carbohydrate and other nutrients necessary for fungal growth.

**Effects of Inducers on Laccase Bioproduction in Flasks.** The laccase bioproduction using *T. versicolor* cultivated on BSG supplemented with different inducers with respect to incubation time was provided in Figure 2A. Higher laccase activities (IU/gds) of 10108 $\pm$ 157.4 and 10006.6 $\pm$ 160.6, respectively, were achieved with phenol (10 mg/kg substrate) and EtOH (20 g/kg substrate) as inducer after 12 days of fermentation period. Similarly, laccase activities (IU/gds) of 5284.8 $\pm$ 168.4 (ABTS, 2 mM), 6216 $\pm$ 164.9 (CuSO4, 2 mM), 5856.9 $\pm$ 136.6 (GA, 1 mM), and 5553.1 $\pm$ 108.6 (VA, 1 mM), respectively, were obtained, which were significantly higher than those of the control (2343.36 $\pm$ 147.9) after 12 days of fermentation time. The decrease in laccase activities was observed after 12 days of incubation time.

Various authors have attempted to increase the production of laccase with white rot fungi by supplementing various inducer compounds, such as caffeic acid, CuSO$_4$, cycloheximide, ethanol, gallic acid, guaiacol, coconut oil, phenol, pyrogallol, soy oil, syringic acid, syringaldazine, tannic acid, veratryl alcohol, and 2,5-xylidine.[15−19] Significantly higher laccase activity of 20-fold more than control was achieved by white rot fungus *T. versicolor* grown on synthetic media supplemented with 10 mg/L phenol.[15] Similarly, authors also achieved higher laccase activities with different inducers.

**Fungal Viability.** The viability (CFUs/g BSG) of *T. versicolor* is provided in Figure 2B. The viability results indicated that maximum growth was obtained around 10−12 days of incubation period. Higher viability (CFUs/g BSG) was obtained in the treatments supplemented with phenol (2.39 $\times$ 10$^8$) and EtOH (2.34 $\times$ 10$^8$). The fungus grows efficiently on solid supports, such as BSG due to high biodegradable load and high moisture content. Moreover, the cultivation of fungi through koji fermentation reproduces the natural living conditions for the fungus. The growth of fungus correlated well with the laccase production trend, as evident from Figure 2B.

**Effect of Inducers on Laccase Bioproduction in Plastic Trays.** Laccase bioproduction was scaled-up in the laboratory using simple and cheap solid-state tray fermentation technology. The comparison of laccase activities obtained in flasks and trays was provided in Figure 3. As compared to the flasks, koji
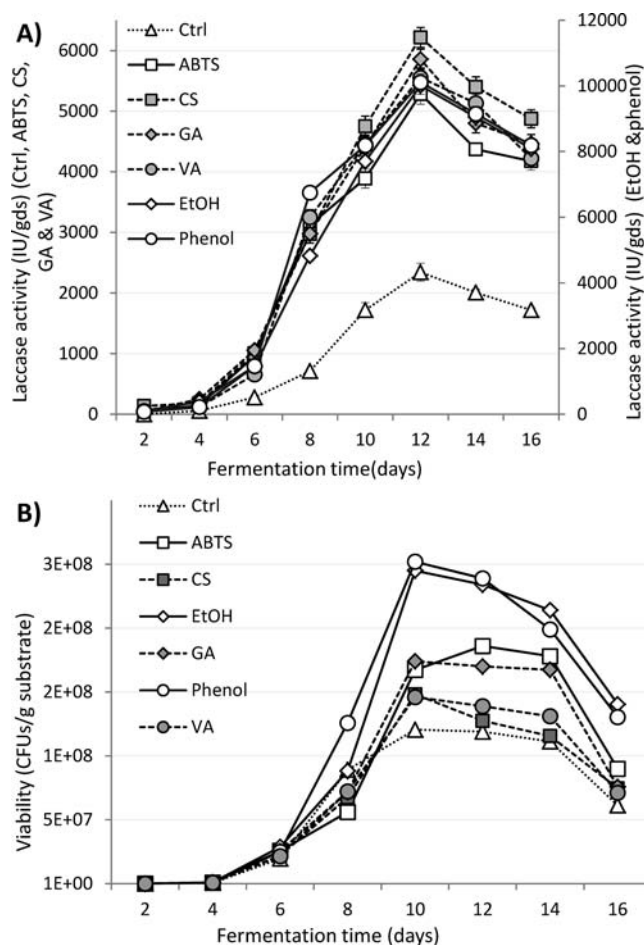


**Figure 2.** (A) Laccase activities and (B) viability of *T. versicolor* cultivated on brewer's spent grain supplemented with different inducers in flasks. The control experiment without any inducer was run in parallel. Viability was determined using the MPN method. All the values given are mean $\pm$ SD, where $n = 3$.
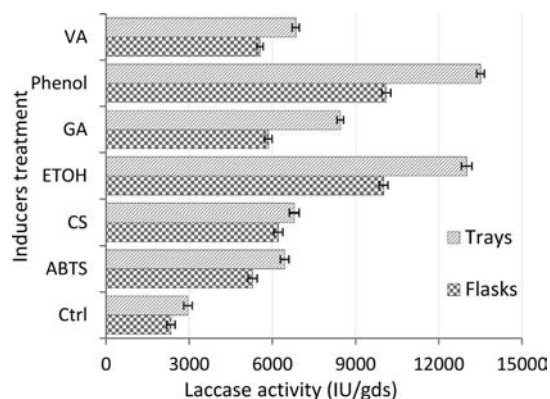


**Figure 3.** Comparison of laccase production (12 days) in flasks and trays by *T. versicolor* cultivated in brewer's spent grain supplemented with different inducers. The control experiment without any inducer was run in parallel.

fermentation in trays resulted in higher laccase activities (IU/gds) as follows: 13506.2 $\pm$ 138.2 (phenol, 10 mg/kg substrate), 13004.6 $\pm$ 190.4 (EtOH, 20 g/kg substrate), 6445.9 $\pm$ 156.1 (ABTS, 2 mM), 6787.2 $\pm$ 173.4 (CuSO4, 2 mM), 8449.4 $\pm$ 120.3 (GA, 1 mM), 6847.7 $\pm$ 126.5 (VA, 1 mM), and 2956.8 $\pm$ 155.3 (control), respectively.

**Table 2. Maximum Laccase Activities Obtained by Different *Trametes* Species in Different Bioreactor Types**

| trametes species | substrate/support | type of bioreactor | inducer | laccase activity (IU/L) | ref |
|---|---|---|---|---|---|
| *T. versicolor* | nylon sponge | immersion (2.5 L) | Tween 80 | 229 | 20 |
| *T. versicolor* | barley bran | immersion (2.5 L) | Tween 80 | 600 | 20 |
| *T. versicolor* | nylon sponge | expanded-bed (300 mL) | Tween 80 | 126 | 20 |
| *T. versicolor* | barley bran | expanded-bed (300 mL) | Tween 80 | 600 | 20 |
| *T. versicolor* | nylon sponge | tray (1 L) | Tween 80 | 343 | 20 |
| *T. versicolor* | barley bran | tray (1 L) | Tween 80 | 3500 | 20 |
| *T. hirsuta* | cuttings of stainless steel sponges + nutrient medium | 0.5 L immersion | $Cu^{2+}$ | 4892 | 11 |
| *T. hirsuta* | nylon sponge | tray (0.2 L) | | 6898 | 21 |
| *T. hirsuta* | grape seeds | tray (0.2 L) | | 18,715 | 21 |
| *T. hirsuta* | grape seeds | immersion (0.5 L) | | 12,877 | 21 |
| *T. hirsuta* | orange peelings | | 5 mM $Cu^{2+}$ | 31,786 | 18 |
| *T. hirsuta* | orange peelings | 500 mL fixed-bed reactor | 5 mM $Cu^{2+}$ | 3000 | 18 |
| *T. hirsuta* | orange peels | 1.8 L tray (200 mL) | 5 mM $Cu^{2+}$ | 12,000 | 18 |
| *T. versicolor* | horticulture waste + mineral salt medium | 200 mL fixed-bed reactor | veratryl alcohol | 8.6 IU/g | 19 |
| *T. versicolor* | BSG | tray (2 L) | 10 mg/kg phenol | 13506.2 ± 138.2 IU/gds | this study |
| *T. versicolor* | BSG | tray (2 L) | 20 g/kg ethanol | 13004.6 ± 190.4 IU/gds | this study |
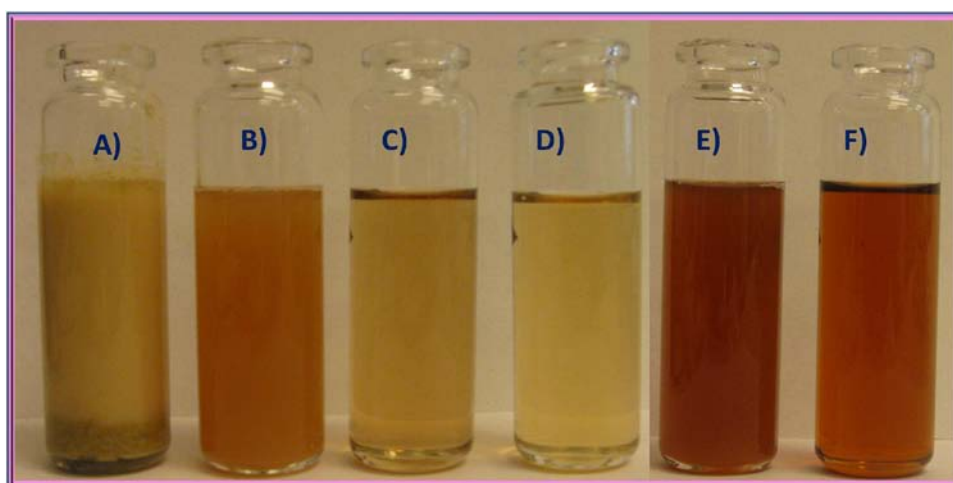


**Figure 4.** Flocculation and clarification of brewery liquor: (A) control-crude beer [simple beer]; (B) after stabifix treatment; (C) laccase treatment (10 IU/L); (D) laccase treatment (25 IU/L); (E) beer (with polyphenols) after stabifix treatment; and (F) after laccase treatment (25 IU/L) after a 48 h incubation time.

The laccase activities achieved in different types of bioreactors with *Trametes* species grown on various solid substrates were given in Table 2. The potential of tray fermentation for laccase bioproduction by *Trametes* species has been demonstrated by various researchers.[18,20,21] Three bioreactor configurations (immersion, expanded-bed, and tray) with different agitation systems (mechanical, pneumatic, and static) for laccase production by *T. versicolor* under SSF conditions using an inert (nylon sponge) and a non-inert (barley bran) supports were evaluated.[20] The authors found that the tray configuration led to the highest laccase activities (Table 2). In one recent study, the authors compared two bioreactor types (immersion and tray) for laccase production by *T. hirsuta* using grape seeds as supports and reported much higher laccase activities in the tray bioreactor.[21] Higher laccase activity was reported in a tray bioreactor compared to a fixed-bed for *T. hirsuta* grown on ground orange peelings.[18]

Koji fermentation in trays is cheap technology and can be potentially used for higher enzyme production. Furthermore, the utilization of low cost agroindustrial wastes without supplementation with expensive media will have important economical and environmental advantages. The laccase production can be further enhanced by optimizing other important process parameters and can be used for the economical production of laccases on a large scale.

**Effect of Laccase on Flocculation and Clarification of Crude Beer.** Visual results of clarification and flocculation of laccase treated crude beer samples are provided in Figure 4. The flocculation properties of the laccase treated crude beer samples have been studied by using various parameters, such as viscosity, turbidity, $\zeta$ potential, total polyphenols, and total protein content, and are discussed below. Overall, improved clarification and flocculation was achieved with the laccase treatment as compared to the industrial treatment with stabifix as a flocculant. The results showed that laccase (25 IU/L) showed better flocculation capacity compared to the industrial flocculation process. The laccase treatments (25 IU/L) at $4 \pm 1$ °C and room temperature have shown almost similar flocculation properties without much variability. Many laccase substrates, such as unsaturated fatty acids, phenols, and thiol-containing proteins, are important constituents of various foods and beverages, including beer. The haze formation in beer is

encountered mainly due to a cross-linking of polyphenols (tannin) causing protein precipitation and by small quantities of naturally occurring proanthocyanidins. The laccase thus oxidizes the phenols which are responsible for haze formation. The downstream processing during industrial fermentations contributes a higher share in the overall cost of the final product. The study demonstrated the potential of in-house produced laccase using brewer's spent grain for the clarification and flocculation of crude beer as a sustainable alternative to traditional flocculants, such as stabifix and bentonite. Laccase mediated clarification and flocculation can efficiently enhance cell broth separation processes for sustainable beer production.

**Viscosity.** The rheological property of the flocculants during flocculation was determined using a rheometer, and the viscosity was measured. The effect of laccase treatment on viscosity was given in Figure 5. The viscosity of the solutions
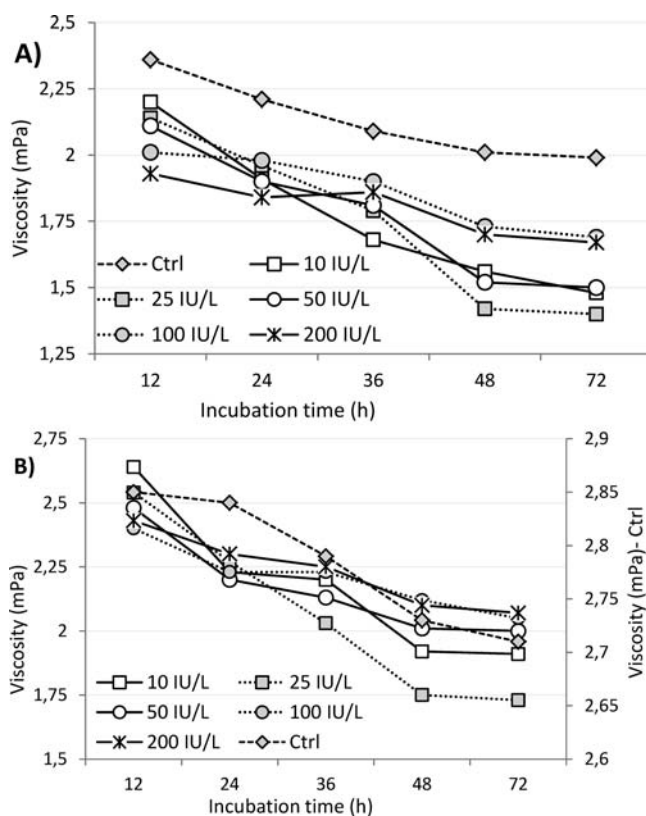


**Figure 5.** Changes in viscosity during laccase treatment (IU/L): (A) beer (simple) and (B) beer (polyphenols) with respect to time at 4 ± 1 °C. The control experiment with heat denatured enzyme was run in parallel. Viscosity after industrial treatment (stabifix): (A) beer (simple), 2.21; and (B); beer (polyphenols), 2.98.

generally decreases with flocculation. The decrease in viscosity was observed in the supernatant during laccase treatment. The viscosity after industrial treatment (stabifix) was observed to be as follows: (1) beer (simple), 2.21; and (2) beer (polyphenols), 2.98, respectively.

The viscosity decreased to 1.42 and 1.52 mPa, respectively in laccase treatment with 25 IU/L and 50 IU/L at 4 ± 1 °C in beer (simple) after a 48 h incubation period (Figure 5 A). Similarly, the viscosity decreased to 1.92 and 1.75 mPa, respectively in laccase treatment with 10 IU/L and 25 IU/L at 4 ± 1 °C in beer with polyphenols after a 48 h incubation time (Figure 5 B). The decrease in viscosity can be attributed to the

settling of the yeast cells and other molecules, such as proteins and polyphenols present in the crude beer after laccase treatment.

**Turbidity.** The effect of laccase treatment on flocculation and clarification of crude beer was given in Figure 6. Turbidity
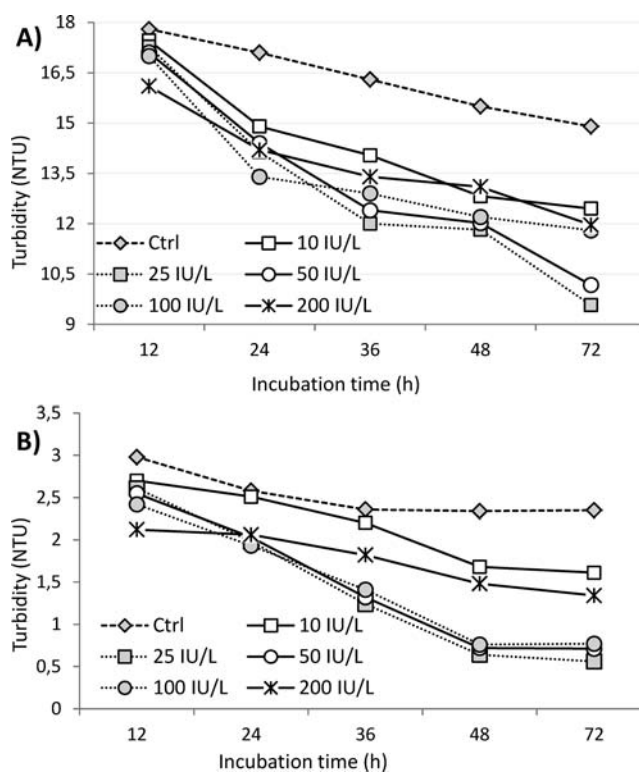


**Figure 6.** Changes in turbidity (NTU) during laccase treatment (4 ± 1 °C) (IU/L): (A) beer (simple) and (B) beer (polyphenols) with respect to time. The control experiment with heat denatured enzyme was run in parallel. Turbidity (NTU): (1) before industrial treatment (crude beer), above the upper limit of detection; and (2) after industrial treatment (stabifix): (a) beer (simple), 1638; and (b) beer (polyphenols), 1038.

after industrial treatment (stabifix) was observed to be as follows: (1) beer (simple), 1638 NTU; and (2) beer (polyphenols), 1038 NTU, respectively. The turbidity decreased during flocculation with laccase treatment. The turbidity was decreased to 11.82 NTU in laccase treatment with 25 IU/L (99.3% reduction in turbidity) and to 12.02 NTU in laccase treatment with 50 IU/L at 4 ± 1 °C (99.2% reduction in turbidity) in beer (simple) samples after 48 h incubation time, as evident in Figure 6A. Similarly, the decrease in turbidity was observed to be 100% in all the beer (polyphenol) samples after 48 h incubation time as compared to the industrial process (Figure 6B). There was no significant difference observed in the turbidity during 48−72 h incubation time ($p < 0.05$). The reduction in turbidity at RT was slightly lower than laccase treatment at 4 ± 1 °C (results not shown).

The main aim of the coagulation/flocculation process in a brewery is the removal of turbidity from the crude brewery liquor. Turbidity is characterized by a cloudy appearance of liquid caused by small particles suspended therein. The end product of a well-regulated flocculation process is that in which the majority of the turbidity has been collected into the floc: clumps of microbial cells and particulate impurities that have come together and formed a cluster. The floc will then settle

out in the sedimentation basin, with remaining floc being removed in the filter. This study demonstrated that the laccase treatment successfully aids in the flocculation of crude brewery liquor.

**ζ Potential.** The charge characteristics of flocculants were characterized by ζ potential. ζ potential is a controlling parameter of double layer repulsion for individual particles and corresponds to the surface charge during the flocculation process.[22] If the ζ potential falls below a certain level, the colloid will aggregate due to the attractive forces. Conversely, a high ζ potential maintains a stable system. It was reported that a decrease in ζ potential led to an increased degree of aggregation or flocculation. DLVO (Derjaguin−Landau−Verwey−Overbeek) theory simply states that the stability of the colloid is a balance between the attractive Van der Waals' forces and the electrical repulsion due to the surface charge.

The ζ potential values during flocculation and clarification of crude beer with laccase are provided in Figure 7. Generally, the
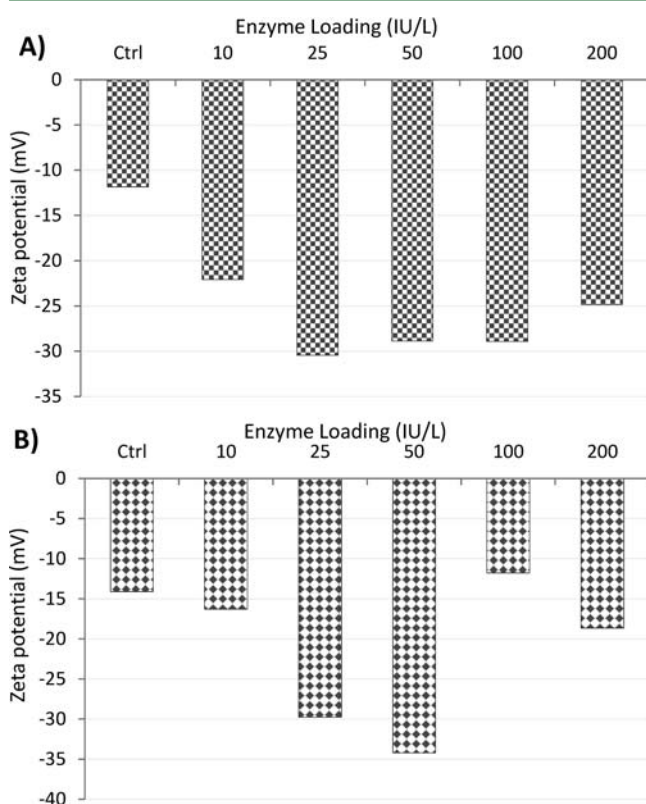


**Figure 7.** Changes in ζ potential (mV) during the laccase treatment (4 ± 1 °C) (IU/L): (A) beer (simple) and (B) beer (polyphenols) after 48 h incubation time. The control experiment with heat denatured enzyme was run in parallel. ζ potential (mV) after industrial treatment with stabifix: (a) beer (simple), 21.94; and (b) beer (polyphenols), 24.20.

ζ potential value decreased with flocculation ability. The ζ potential in the control samples without flocculation was in the range −5.95 to −23.35 mV. The ζ potential was decreased to −30.45 mV in laccase treatment with 25 IU/L, and −28.88 mV in laccase treatment with 50 IU/L at 4 ± 1 °C after 48 h (Figure 7A). Similarly, during laccase treatment at RT, the ζ potential values of −26.24 and −19.04 mV were recorded in a beer (simple) sample after 48 h incubation time. Likewise, the ζ potential was decreased from −29.78 to −34.21 mV in the beer

(polyphenols) sample with laccase treatments of 25 and 50 IU/L, respectively (Figure 7B).

**Total Polyphenolics Content.** Figure 8 represents the polyphenol content during laccase treatment of crude beer
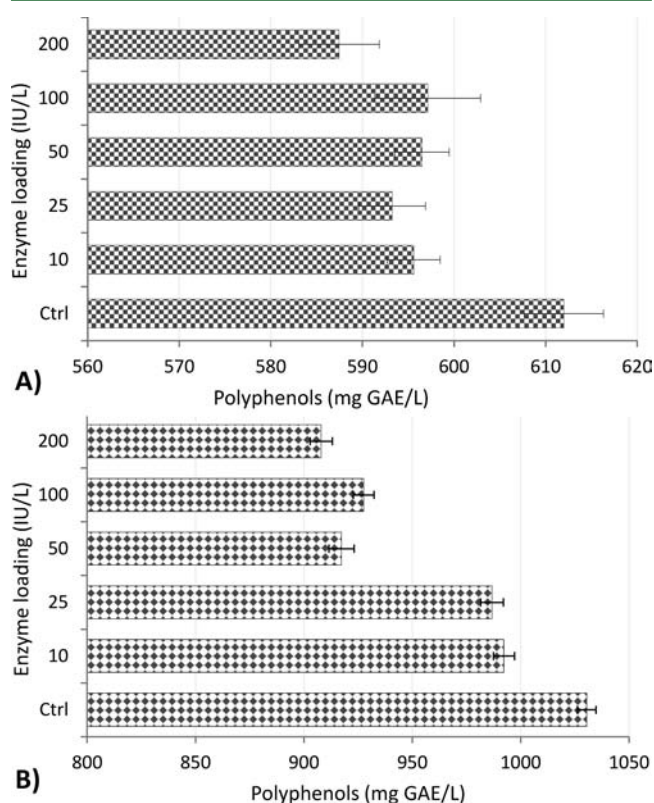


**Figure 8.** Total polyphenol content during the laccase treatment (4 ± 1 °C) (IU/L): (A) beer (simple) and (B) beer (polyphenols) after 48 h incubation time. The control experiment with heat denatured enzyme was run in parallel. Total polyphenol content (mg GAE/L) after industrial treatment (stabifix): (A) beer (simple), 730.5 ± 18.5; and (B) beer (polyphenols), 1052.9 ± 27.8.

samples. The polyphenol content in beer (simple) samples during laccase treatment was 593.2 ± 2.9 and 596.5 ± 3.6 mg GAE/L in treatment with 25 and 50 IU/L, respectively, at 4 ± 1 °C after 48 incubation time (Figure 8A). Similarly, the polyphenol content in beer (simple) samples during laccase treatment was 625.3 ± 3.2 and 601.6 ± 6.3 mg GAE/L, respectively, with 25 and 50 IU/L at RT after 48 incubation time. There was no significant difference in polyphenols content during 48−72 h incubation time ($p < 0.05$). Likewise in the beer (polyphenols) sample, the polyphenol content was recorded as 986.8 ± 4.8 and 917.3 ± 5.1 mg GAE/L in treatment with 25 IU/L and 50 IU/L at 4 ± 1 °C after 48 incubation time (Figure 8B). The polyphenol content was observed to be 993.2 ± 3.9 and 922.1 ± 4.7 in treatment with 25 and 50 IU/L at RT after 48 h incubation time. The polyphenol content in the beer (simple) and beer (polyphenol) after industrial treatment was observed to be 730.5 ± 18.5 and 1052.9 ± 27.8 mg GAE/L respectively. There was not much reduction in the polyphenol content during flocculation with laccase treatment as compared to control. The overall decrease in total polyphenol content may be due to the complex formation with other biomolecules, such as protein during flocculation and settling.

The beer contains a complex mixture of phenolic compounds extracted from malt and hops which have been shown to have useful antioxidant properties.[23] Moreover, three groups of polyphenols are responsible for beer flavor and physical stability as described by a number of authors.[23−25] Simple polyphenols derived from hydroxybenzoic acids (gallic acid, protocatechuic acid, etc.) and hydroxycinnamic acids (ferulic acid, p-coumaric acid, caffeic acid, etc.) are extracted mostly from malt but are also present in small amounts in hops. Flavonols (quercetin, kaempferol, etc.) come mostly from hop. Flavan-3-ols, including monomers such as (+)-catechin and (−)-epicatechin dimers (prodelphinidin B3 and procyanidin B3), trimers (procyanidin C2), and flavonoid-derived tannins up to higher molecular weights, arise equally from malt and hop. The final content of phenolic components of beer depends on both the raw materials and the brewing process. It is evident from the present results that clarification was much better as compared to the industrial samples treated with stabifix along with the preservation of polyphenolic content, which is sought for the flavor and quality of the final products.

**Total Protein Content in Laccase Treated Brewery Liquor.** Proteins contained in beer are for the most part derived from water-soluble proteins contained in the grains used in the malting and brewing process. These proteins are important determinants of beer quality, yet most proteins are modified or lost during the malting process. The protein content during laccase treatment of crude beer was given in Figure 9. The protein content in the fermented liquor without
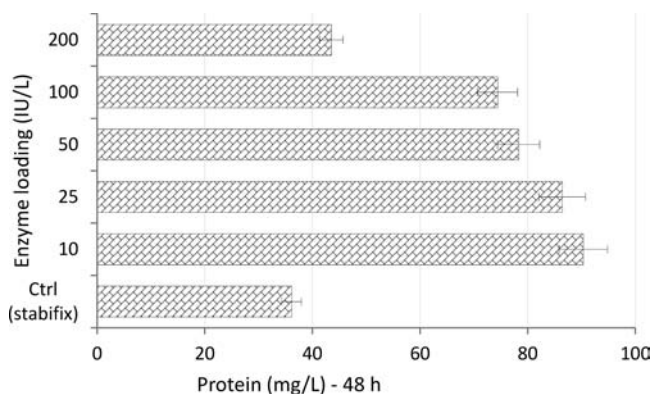


**Figure 9.** Changes in protein content in beer (simple) samples during the laccase treatment (4 ± 1 °C) after 48 h incubation time. (During laccase treatment, the enzyme concentration was also taken into consideration during protein estimations.) Total protein content (mg/L) in: (1) crude beer (simple), 194.04; and (2) after industrial treatment (stabifix in beer (simple), 36.12.

flocculation was in the range of 194.04 mg/L of the liquor and after industrial treatment (stabifix) was 36.12 mg/L. This decrease in protein content during flocculation may be due to the settling of yeast cells and other protein present in the malt and hops. The protein content during flocculation decreased to 86.4 and 78.32 mg/L, respectively, in laccase treatment with 25 and 50 IU/L at 4 ± 1 °C (Figure 9) and 78.54 and 71.75 mg/L, respectively, in laccase treatment with 25 and 50 IU/L respectively, at RT in beer (simple) samples after 48 h incubation time. The protein content was higher in the laccase treated beer (simple) samples as compared to industrial sample treated with stabifix, which increases its nutritional value. However, during laccase treatment, the enzyme concentration

was also taken into consideration for protein estimation. Hence, the enzyme does not contribute to the increase in protein content. In industrial treated samples with stabifix, it is possible that the protein was removed during settling, which resulted in reduced protein content in the processed sample. The protein content observed was similar to the industrial treatments in beer (polyphenols) samples and was not detectable.

**Preliminary Cost Calculations Using Enzymes for Flocculation and Clarification.** The costs associated with clarification and flocculation of fermented brewery liquors are provided in Table 3. The preliminary cost calculations were

**Table 3. (A) Estimated Cost Price of Different Treatments Used for Clarification and Flocculation of Brewery Liquor and (B) Preliminary Cost Analysis of Clarified Brewery Samples with Laccase**

| (A) commodity (quantity) | price ($) | quantity used |
|---|---|---|
| stabifix (20 kg) | 119 | 0.05% (w/v) |
| bentonite (20 kg) | 19.25 | 0.015% (w/v) |
| laccase (1000 IU) | 104.15 | 25 IU/L |

| (B) treatment | required amount/year | total costs ($)/year | working time (days) |
|---|---|---|---|
| stabifix | 0.5 g/L per year = 0.5 × 14000 L × 30 (batch/year) = 210 kg | 1249.50 | 300 |
| bentonite | 0.15 g/L per year = 0.5 × 14000 L × 30 (batch/year) = 63 kg | 60.64 | 300 |
| laccase (commercial source) | 25 IU/L per year = 25 × 14000 L × 30 | 1093575 | 60 |
| in-house produced laccase | | 437430 | 60 |

performed in this study to compare the traditional flocculation and clarification using bentonite and stabifix and using laccase enzymes. The beer industry in this case operates 14 fermentors (1000 L capacity) for clarification and flocculation of fermented broth, which takes 10 days/batch. Assuming the industry works 300 days/year, a total of 30 batches can be completed/year. The industry operates 14 fermenters of 1000 L capacity with 30 batches/year, which is an equivalent of 420,000 L of beer annually. It was assumed that the cost of in-house produced enzyme using brewers spent grain (BSG is the byproduct generated by the beer industry after mashing of grains) will be 40% less than the commercial sources. As evident from results, only slight differences were observed using 10 IU/L and 25 IU/L and at 4 ± 1 °C and RT, respectively, during laccase treatment. The concentration of crude enzymes used for clarification and flocculation can be reduced significantly by purification of enzyme, amendment with biopolymers, such as chitosan, and optimization of other important factors, such as temperature. Due to its cationic nature, chitosan binds to the positively charged yeast cells and other biomolecules and therefore can act synergistically with the laccase enzyme. It will also solve the problem of waste management, as currently BSG was not used for the formation of any value added products.

The comparison of costs associated with clarification of fermented brewery liquor with traditional methods and with in-house produced laccase is given in Table 3. The clarification of fermented brewery liquor with laccase (48 h/batch) will be completed in 60 days as compared to traditional methods (300 days/year). This will result in a significant decrease in the time as well as other costs associated with manpower, energy, and

chemicals. This time can be utilized for processing of more fermented brewery liquor that will expand the industrial capacity and will generate extra revenues for the industry. The study demonstrated the potential of laccase for efficient flocculation of crude beer, indicating that laccase treatment could lead to significant improvements in the processing of biotechnological fermentation products, such as beer and wine production. Laccase can be used as a clarifying and flocculation agent in the beer industry as an alternative to physical–chemical adsorbents, such as bentonite.

Beer clarification is one of the most important operations, when rough beer is filtered in order to eliminate yeast and colloidal particles responsible for haze. In addition, this operation should also ensure the biological stability of the beer. The flocculation properties of the different laccase treated brewery fermented liquor samples have been studied by using various parameters, such as viscosity, turbidity, $\zeta$ potential, total polyphenols, and total protein content. The results showed that laccase (25 IU/L) showed promising results as a good flocculating agent. The concentration of laccase can be further reduced, as only slight differences were observed during flocculation and haze removal from crude brewery liquor with 10 and 25 IU/L. The laccase treatment showed improved flocculation [beer (simple) 99.2% and beer (polyphenols) ~100%] capacity compared to the industrial flocculation process using stabifix. The laccase treatments at 4 ± 1 °C and room temperature have shown almost similar flocculation properties without much variability. Further studies need to be carried out to quantify the concentration of laccase by using the statistical approach of response surface methodology. Being energy-saving and biodegradable, laccase-based biocatalysts fit well with the development of highly efficient, sustainable, and ecofriendly industrial processes. Thus, use of laccase helps in promoting odor control, taste enhancement, or reduction of undesired products in beer.

## ■ AUTHOR INFORMATION

### Corresponding Author
*Phone: +1 418 654 3116. Fax: +1 418 654 2600. E-mail: satinder.brar@ete.inrs.ca.

### Notes
The views or opinions expressed in this article are those of the authors.
The authors declare no competing financial interest.

## ■ ABBREVIATIONS

ABTS-2, 2-azino bis(3-ethylbenzthiazoline-6-sulfonic acid); ANOVA, analysis of variance; ATCC, American type culture collection; BSG, brewer's spent grain; CFUs, colony forming units; CS, CuSO$_4$; EtOH, ethanol; GA, gallic acid; GAE, gallic acid equivalents; gds, gram dry substrate; MPN, most probable number; NTU, nephelometric turbidity units; RT, room temperature; SD, standard deviation; SSF, solid state fermentation; VA, veratryl alcohol

## ■ REFERENCES

(1) Kirk, T. K.; Fenn, P. Formation and action of the ligninolytic system in basidiomycetes. In *British Mycological Society Simposium 4, Decomposer basidiomycetes*; Franland, J. C., Hedger, J. N., Swift, M. J., Eds.; Cambridge University Press: 1982; pp 67–90.

(2) Lorenzo, M.; Moldes, D.; Rodriguez-Couto, S.; Sanroman, A. Improving laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. *Bioresour. Technol.* **2002**, *82*, 109–113.

(3) Moll, M. *Bieres et coolers e definition, fabrication, composition*; Tech & Doc Lavoisier: Paris, 1991; Chapter III, pp 15–263.

(4) Stewart, G. G.; Russel, I. Yeast flocculation. In *Brewing Science*; Pollock, J. R. A., Ed.; Academic Press: New York and London, 1982; Vol. 2, pp 61–92.

(5) Verstrepen, K. J.; Derdelinckx, G.; Verachtert, H.; Delvaux, F. R. Yeast flocculation: what brewers should know? *Appl. Microbiol. Biotechnol.* **2003**, *61* (3), 197–205.

(6) Caridi, A. Enological functions of parietal yeast mannoproteins. *Antonie van Leeuwenhoek* **2006**, *89* (3–4), 417–422.

(7) Zhao, X. Q.; Bai, F. W. Yeast flocculation: new story in fuel ethanol production. *Biotechnol. Adv.* **2009**, *27* (6), 849–856.

(8) Eggert, C.; Temp, U.; Eriksson, K. E. L. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of laccase. *Appl. Environ. Microbiol.* **1996**, *62*, 1151–1158.

(9) Lee, I.-Y. Y.; Jung, K.-H. H.; Lee, C.-H. H.; Park, Y.-H. H. Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. *Biotechnol. Lett.* **1999**, *21*, 965–968.

(10) Rodrıguez-Couto, S.; Gundın, M.; Lorenzo, M.; Sanroman, M. A. Screening of supports and inducers for laccase production by *Trametes versicolor* in semi-solid-state conditions. *Proc. Biochem.* **2002**, *38*, 249–255.

(11) Rodríguez-Couto, S.; Hofer, D.; Sanromán, M. A.; Gübitz, G. M. Production of laccase by *Trametes hirsuta* grown in an immersion bioreactor. Application to decolourisation of dyes from a leather factory. *Eng. Life Sci.* **2004**, *4*, 233–8.

(12) Dhillon, G. S.; Brar, S. K.; Kaur, S.; Valero, J. R.; Verma, M. Chitinolytic and chitosanolytic activities from crude cellulase extract produced by *A. niger* grown on apple pomace through koji fermentation. *J. Microbiol. Biotechnol.* **2011**, *21* (12), 1312–1321.

(13) ISO 14502-1. Determination of substances characteristic of green and black tea. Part 1: Content of total polyphenols in tea. Colorimetric method using Folin-Ciocalteu reagent; 2005.

(14) AOAC. *Official methods of analysis method 988.05*; AOAC International: Gaithersburg, MD, 1999a; Chapter 4, pp 13.

(15) Pazarlioglu, N. K.; Sariisik, M.; Telefoncu, A. Laccase: production by *Trametes versicolor* and application to denim washing. *Proc. Biochem.* **2005**, *40*, 1673–1678.

(16) Gnanamani, A.; Jayaprakashvel, M.; Arulmani, M.; Sadulla, S. Effect of inducers and culturing processes on laccase synthesis in *Phanerochaete chrysosporium* NCIM 1197 and the constitutive expression of laccase isozymes. *Enzyme Microb. Technol.* **2006**, *38*, 1017–1021.

(17) Minussi, R. C.; Rossi, M.; Bologna, L.; Rotilio, D.; Pastore, G. M.; Duran, N. Phenol removal in musts: Strategy for wine stabilization by laccase. *J. Mol. Catal. B: Enzym.* **2007**, *45*, 102–107.

(18) Rosales, E.; Rodrıguez-Couto, S.; Sanroman, M. A. Increased laccase production by *Trametes hirsuta* grown on ground orange peelings. *Enzyme Microb. Technol.* **2007**, *40*, 1286–1290.

(19) Xin, F.; Geng, A. Utilization of horticultural waste for laccase production by *Trametes versicolor* under solid-state fermentation. *Appl. Biochem. Biotechnol.* **2011**, *163*, 235–246.

(20) Rodríguez-Couto, S.; Moldes, D.; Liébanas, A.; Sanromán, Á. Investigation of several bioreactor configurations for laccase production by *Trametes versicolor* operating in solid-state conditions. *Biochem. Eng. J.* **2003**, *15*, 21–6.

(21) Rodríguez-Couto, S.; López, E.; Sanromán, M. A. Utilisation of grape seeds for laccase production in solid-state fermentors. *J. Food Eng.* **2006**, *74*, 263–267.

(22) Rasterio, M. G.; Garcia, F. A. P.; Ferreira, P.; Blanco, A.; Negro, C.; Antunes, E. Evaluation of flocs resistance and reflocculation capacity using the LDS technique. *Powder Technol.* **2008**, *183* (2), 231−238.

(23) Goupy, P.; Hugues, M.; Boivin, P.; Amiot, M. J. Antioxidant compounds of barley (*Hordeum vulgare*) and malt extracts. *J. Sci. Food Agric.* **1999**, *79*, 1625−1634.

(24) Shahidi, F.; Naczk, M. *Food Phenolics: Sources, Chemistry, Effects, Applications*; Technomic Publishing Co. Inc.: Lancaster, 1995; pp 10−13.

(25) Čepička, J.; Karabin, M. Polyfenolove latky piva—přirozene antioxidanty. *Chem. Listy* **2002**, *96*, 90−95.