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Value-Added Use of Waste Mushroom Stipes Production of Fermented Alcoholic Beverage

Evan Wright[1], Lane Harper[1], Juan Carrera Rivas[1], Ping Wu[1,2]

1. School of Engineering, University of Guelph, Guelph, Ontario, Canada, N1G 2W1
2. Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph, Ontario, N1H 4Y2

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ABSTRACT Fermentation is an important biotechnology for the manufacture of alcohol for both fuel and consumption. Nitrogen and other key nutrients have a large influence on the fermentation rate and the final product quality. Mushroom stipes (stems) is a 13 million kg a year resource currently used for low value applications such as compost or animal feed. The stipes are high in many nutrients including minerals, vitamins, and amino acids. They also contain many functional compounds with a wide range of claimed health benefits. This experiment seeks to examine whether mushroom stipe extract can be used as a nutrient source to facilitate the growth, fermentation, and subsequent ethanol production of *Saccharomyces cerevisiae* a strain typically used for white and sparkling wines. The final product is a drinkable alcoholic beverage comparable in ethanol content to a typical white wine.

Keywords: food waste, mushroom stipes, fermentation, ethanol, Saccharomyces cerevisiae

INTRODUCTION Increasing amounts of food waste is a problem that is becoming more and more relevant in modern times. According to Gooch and Felfel (2014), the quantifiable amount of Canada's annual food waste is approximately 31 billion dollars. Even more daunting is that this was just the quantifiable value, with the true value being much higher due to associated costs in generating the waste, including: energy, water, land, labour, capital investment, infrastructure, machinery, and transport, among others. Furthermore, in Canada it is estimated that 30% of food waste occurs at the farm and processing level (Gooch and Felfel 2014). One commodity which is a perfect example of this problem is mushrooms. The cultivation of mushrooms in Canada is a 370 million dollar industry producing approximately 111,000 tonnes per year, with Ontario alone producing over 50% of that amount (Statistics Canada 2014). Of that total, approximately 90% of the mushrooms grown are the species Agaricus bisporus, which includes White Button mushrooms as well as brown mushrooms such as Cremini and Portabella. As part of the cultivation of A. bisporus, typically the bottom of the stem (the stipe) is discarded by the growers or used as low economic value animal feed or compost (Chou 2013). It is estimated that the discarded stipe composes 25-30% of the harvested mushroom (Ndungutse 2015), meaning that the stipes of A. bisporus account for over 25,000 tonnes each year of waste.

Mushroom stipes are an incredibly versatile product containing many of the same characteristics and chemical compounds as the mushroom itself, meaning that there are many potential uses for the stipe. One such use is as a nutritional source or supplement. The stipes of A. bisporus contain a wide range of vitamins and minerals such as vitamin C, vitamin E, phosphorus, manganese, iron, magnesium, and others. They are also a significant source of protein and fibre (Poongkodi 2015). Based on this, a potential way to reduce the waste from mushroom stipes is to use them in biotechnology and bioprocess application as a nutrient source. One such potential area is in the fermentation of simple sugars into ethanol by yeast, one of the most prevalent and oldest bioprocesses. The stipes could serve as an additional nutrient source in the fermentation medium to enhance the growth rate of the yeast and increase ethanol product; and this could potentially have large implication in both biofuels and beverages.

Ethanol is the most utilized biofuel in the world and it is also the main biofuel used for transportation with fuel ethanol representing almost 50% of the total fuel volume consumed by cars and light vehicles. (Basso et al. 2011). Due to the fact that ethanol is used as biofuel, it can be generated from the same biotechnological processes that are used in creating alcoholic beverages, namely fermentation. However, there are slight differences in the post-fermentation processing of a simple fermented beverage like wine and the pure ethanol that causes it's alcohol content. Typically, the industrial process (for fermentation and subsequent distillation) follows a rather simple, routine procedure. The first step is selection of a feedstock that supplies sugars and nutrients required for bacterial fermentation. In the creation of an alcoholic beverage, the feedstock is an important consideration as it can influence the final flavours of the beverage and most often the whole feedstock is used (i.e. whole corn) versus an agricultural waste product such as stems, stalks, or other organic, nutrient containing waste products. However, ethanol can be produced from a large selection of renewable feedstock that can be grouped into three main categories: those containing large quantities of easily fermentable sugars, starches and fructosans, and cellulosics (Basso et al. 2011). The choice of feedstock has a huge effect on ethanol production costs, with very little cost-effectiveness in using good agricultural land to grow large swaths of monocrops to be used in fermentation processes. The next step is the fermentation process itself. This is can be done in a variety of different bioreactors, utilizing different methodologies such as fed-batch, stirred tank, or continuous flow, and with a variety of different microorganisms. However, yeast fermentation is heavily studied and traditionally the most utilized throughout history. In recent times, there has been an increase of interest in other microorganisms and in genetically engineered organisms (including

yeast) to increase yields and slightly change the properties of the final product. The entire fermentation process will be discussed in detail in a separate subsection of the paper

Based on a literature review, very little work has been done on the potential of mushroom stipes as an added fermentation nutrient source, with no work at all being found relating to biofuels. A single study by Lin (2010) investigated using a mushroom stipe extract and cane sugar to create a fermented alcoholic beverage. Their results showed a high final ethanol concentration and a final product which was deemed similar in consumer appeal to commercial white wine (Lin 2010). Based on the concepts methods of this study, this report will similarly investigate the viability of mushrooms stipes for the production of a fermented beverage of high alcohol concentration. In addition, control trials will be run to demonstrate the enhanced fermentation provided by the stipe extract.

The decision to make a beverage was based in this experiment on proving the ability of mushroom waste to increase fermentative yield from an easily obtainable yeast species and to obtain a useful product. To continue to biofuel would only require a distillation procedure on the final fermented product. However, as far as adding value to waste products, creation of an alcoholic beverage would be a very promising route. According to Statistics Canada (2015), Canadians spent $20.5 billion on alcoholic beverages in the 2013/2014 fiscal year, with $6.4 billion total in wine purchases. Creating a novel, interesting wine-type beverage from a non-traditional source could attract many potential customers curious to try something new. To determine if Canada is a suitable marketplace for a "mushroom wine" proper market research would need to be conducted.

FERMENTATION AND METABOLISM BACKGROUND

Fermentation is the chemical process by which glucose molecules are broken down anaerobically to produce ethanol and carbon dioxide gas. It is a widely used process in the wine and beer industries by the use of yeast or other microorganisms. Fermentation has been used by our ancestors dating back to ancient china around 7,000 B.C. which chemical analysis revealed of a fermented drink out of honey, rice and fruit in pottery jars according to McGovern (2004). Microbiologist Louis Pasteur was the first to describe the fermentation process as the breakdown of glucose molecules into ethanol by living organisms such as yeast, and not by decay, set in motion by the presence of dead nitrogenous material like it was previously thought (Thayer & Vallery-Radot 1923). Moreover, fermentation has been used for the preservation and enhancement of foods and beverages throughout history due to the analgesic and disinfectant properties of alcohol.

The process begins with the main reactions of glycolysis which takes place in most cells to break down glucose into 2 moles of ATP (Adenosine Trisphosphate) and 2 moles of pyruvate per mole of glucose (Barnett & Barnett 2011). Fermentation is not only done by microorganisms, prokaryotes and eukaryotes but is also seen in plant cells and even muscle cells in the human body when the amount of oxygen is limited. While the glycolysis pathway is known to be universal among all organisms, microorganisms have the ability to process pyruvate through many different pathways, depending on many factors, to give rise to a multitude of end products (El-Mansi et al. 2012). Figure 1, shows the diversity of different fermentation pathways among microorganisms.

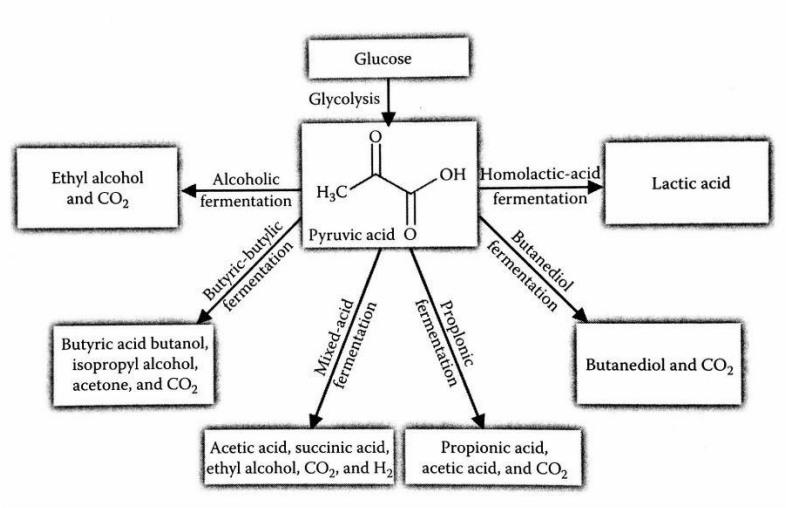

Figure 1. The diversity of fermentation pathways among microorganisms (El-Mansi et al. 2012)

Any living organism needs a supply of nutrients in order to grow and survive. These nutrients are also the building blocks that such organisms are made of. Bamforth (2005) describes organotrophs as organisms capable of oxidizing organic molecules, most commonly sugars. Microorganisms have a great demand for carbon, hydrogen and oxygen and these three of the main four elements (carbon, hydrogen, oxygen and nitrogen) are found in microorganisms' main source of food, sugars. Oxygen is also essential for aerobic respiration, which some organisms grow by such process. However, some organisms are not limited to oxygen for growth and can sustain themselves through the anaerobic process of fermentation. When oxygen is limited (anaerobic pathway), fermentation happens and the end products are carbon dioxide and ethyl alcohol. Meanwhile, when oxygen is present (aerobic pathway), oxidation of sugar happens to form carbon dioxide and water. It begins with glycolysis where pyruvate then goes into the tricarboxylic acid (TCA) to be broken down into a series of organic acids and then ADP is phosphorylated in the electron transport chain to produce ATP and water.

Sugar is not the only nutrient microorganism need as it only provides carbon, hydrogen and oxygen to the cell. In order to grow, microorganisms require carbon, hydrogen, oxygen and nitrogen as they are the elemental constituents of key cellular components such as carbohydrates, lipids, proteins and nucleic acids, so phosphorus and sulphur are also important in this regard (Bamforth 2005). Nitrogen is a fundamental component of protein, and as Bamforth (2005) mentions, most cells are comprised of 15% protein by weight. Yeast growth is strongly influenced by nitrogen as well as its metabolism during fermentation since nitrogen deficiency is believed to be the major cause of stuck or sluggish fermentation (Beltran et al. 2005).

The growth of microorganisms can be affected by various parameters including temperature, pH, water activity, oxygen, among others. Microorganism are very peculiar on the environment they strive in. High temperatures cause denaturation of enzymes in some microorganism due to heat, especially *S. cerevisiae*, a yeast that is considered a mesophile. Mesophiles are organisms which prefer the less extreme temperature ranges at about 10 – 40°C (Bamforth, 2005). Another parameter which range is very specific to the growth of some microorganisms is pH. It affects the ability of the cells to maintain the desired intracellular pH and the charge status of enzymes as it affects their ability to perform (Bamforth, 2005). Water activity is a major factor as some organisms do not grow at a water activity below 0.9 since the majority of microorganisms are comprised of 80% water. As for oxygen, it is required for respiration and to perform certain cellular activities,

however *S. cerevisiae* can perform either aerobic respiration or anaerobic fermentation. It the case of fermentation for the production of alcohol, anaerobic fermentation is the goal.

Saccharomyces cerevisiae is commonly known as the baker's yeast or brewer's yeast as it is the standard yeast used in wine brewery and making bread. The concentration of sugar that the yeast is exposed to impacts the form of metabolism in the cell (aerobic respiration or fermentation). According to Bamforth (2005), high concentration of sugar switches the cell into fermentation mode since the cell does not need to generate as many ATP per glucose molecule due to the excess of sugar. Fermentation by yeast can be enforced by limiting the oxygen supply (anaerobic environment) and having a high concentration of sugar.

The choice of the exact organism to be used for fermentation can greatly change the outcome and the characteristics of the final product. When creating a fermented beverage, yeast of the genus Saccharomyces is almost always used, however there are many different species. *S. cerevisiae* is often considered the standard yeast, and it is used for wine, beer and bread. *S. bayanus* is occasionally used in wine and cider making, *S. paradoxus* is a wild yeast for traditional meads or cider and *S. pastorianus* is used for lager (Fix 1999). While all of these organisms anaerobically convert sugar to ethanol, they can have different fermentation characteristics such as ideal temperature, alcohol tolerance, acid tolerance, and by-products affecting flavour.

Furthermore, each species can have a wide variety of strains, again each with different characteristics. Just within *S. cerevisiae* there are hundreds of distinct strains each with their own unique properties. The strain used in this experiment, EC-1118 was selected based on its fast fermentation rate and high alcohol tolerance of up to 18%. In addition, it is known for high glycerol production, and low acetaldehyde and hydrogen sulphide production (Lallemand 2016).

BIOREACTOR SETUP

The bioreactor vessel used in this experiment consists of a 1 gallon (3.79 L) jug to be used for batch fermentation. The top of the jug is sealed with a rubber stopper and an airlock. The product to be fermented is placed inside the jug along with yeast to inoculate it and the jug is sealed for the entire fermentation process.

When designing a bioreactor vessel for fermentation there a multiple factors to consider. Temperature and heat transfer will greatly influence the growth rate and metabolism of the yeast. The strain used in this experiment, EC-1118, has a recommend growth range of 10 to 30°C (Lallemand 2016) and so the temperature of the bioreactor must be maintained within this range. In many large scale and commercial breweries active cooling methods must be implemented as the heat generated by metabolic activity will cause it to overheat in the large vessels with low surface area to volume ratios. To determine if this is necessary for the bioreactor used in these experiments, the rate of heat generation can be compared to the rate of heat loss when the bioreactor is at 30°C; if the rate of heat loss is greater than the heat generation, then 30°C, the upper limit for ideal growth temperature, would not be reached by the system.

By performing a stoichiometric heat balance on the growth of *S. cerevisiae*, it can be found that the rate of heat generation is 8.1kJ/mol glucose (Uzir 2007). Based on the work of Lin (2010), the maximum rate of glucose utilization was 22.5g/L*day. Given the bioreactor will be filled with 3L of liquid, and knowing the molar mass of glucose to be 180.16g/mol, it can be estimated that the maximum rate of heat generated will be only 0.035W. The rate of heat loss from bioreactor at 30°C can be calculated assuming that natural convection is the main mechanism of heat loss (Calculations not shown). This gives a rate of heat loss of 0.208W, which is a full order of magnitude greater than the rate of heat generation. It is therefore safe to assume that the system will not need any active cooling to remain below 30°C.

Another consideration in designing the bioreactor is managing the production of gasses, specifically CO2; if the vessel were fully sealed the pressure and concentration would build up inside. In some cases this is advantageous, such as the production of sparkling wine and carbonated beer. Carbonation is not part of this protocol however and so the gas must be released without allowing oxygen to enter and cause aerobic metabolism. To accomplish this a one-way airlock which allows CO2 to escape is used.

While not traditionally implemented in bioreactors for alcoholic beverage fermentation, agitation and stirring has been implemented in some systems to increase the rate of fermentation (Nienow 2011). On larger scales, bioreactor may become non-homogeneous, and varying nutrient, dissolved gasses, and ethanol concentrations can lower the maximum rate of fermentation. For the purpose of this experiment, it is assumed that the bioreactor is small enough that convective mixing and agitation from bubble formation will be sufficient.

The final design parameter to consider is the size of the bioreactor, as it will decide the volume of experiment while also having a great effect on other parameters such as heat and mass transfer. For a small scale experiment, a small 1 gallon (3.79L) jug is sufficient. As a general rule when fermenting alcoholic beverages, the jug should only be filled to 80-85% of its maximum volume (Fix 1999). This extra space gives room for any potential foam which is formed during the fermentation and prevents the bioreactor from foaming over. For the 3.79L jug, 3L of liquid is just under 80% and so it is a suitable volume to work with.

EXPERIMENTAL PROTOCOL

The purpose of this experiment was to investigate if mushroom stipes could be used as a nutrient source for the anaerobic fermentation of *S. cerevisiae*, potentially providing an increased rate or increased total amount of ethanol production. This will be assessed by comparing fermentation with a mushroom extract as the liquid medium to fermentation with only distilled water as the liquid medium.

All mushroom stipes were obtained fresh from Monaghan Farms in Milton, Ontario and were of the variety Agaricus bisporus. The stipes were washed and divided into small pieces, then immediately used. Following the work of Lin (2010), 3g/100ml of the stipes were boiled in distilled water for 10 minutes to create a mushroom stipe extract. This extract was used to create 3 replicates, each consisting of 3L of the mushroom stipe extract and 20g/100ml of sucrose. A control of 3L of distilled water and 20g/100ml of sucrose was also prepared. The four solutions were placed into 1 gallon jugs and autoclaved.

To activate and prepare the yeast, a solution of 20g of sugar in 100ml of distilled water was prepared in an Erlenmeyer flash and then autoclaved. Once cooled, one 5g commercial package of EC-1118 *S. cerevisiae* was added and left to grow for 24 hours. This process allows the yeast to begin growing and overcomes the lag phase so that it will have a high growth rate as soon as it is added to the bioreactor.

The bioreactors were each inoculated using 10ml of the activated yeast solution and then sealed with the rubber stopper and airlock. The bioreactors were left at ambient temperature and allowed to ferment undisturbed for 7 days, after which all measurements were taken. A summary of the 4 trials is shown below in Table 1. The process was then repeated, with the only difference being a 14 day fermentation period.

Table 1. Summary of the 4 fermentation trials performed

	Replicate 1	Replicate 2	Replicate 3	Control
Liquid Medium	3L mushroom extract	3L mushroom extract	3L mushroom extract	3L distilled water
Carbon Source	20g/100ml sucrose	20g/100ml sucrose	20g/100ml sucrose	20g/100ml sucrose
Yeast	10ml of activated solution	10ml of activated solution	10ml of activated solution	10ml of activated solution

The measurements performed prior to fermentation were protein content and polysaccharide content, and the measurements preformed upon finishing fermentation were alcohol content, pH, protein content and polysaccharide content.

To measure protein content, a commercial Pierce™ BCA Protein Assay Kit was used. In this test method, protein in an alkaline environment reduces Cu2+ to Cu1+, which is then detected through a colorimetric reaction with bicinchoninic acid. The colour of the final solution can then be compared to know standards to determine the protein concentration (Thermo Scientific 2015). The solution to be tested, either mushroom broth or the final fermentation product, is diluted 1:10 and then tested following the standard protocol.

For polysaccharide content, the phenol-sulphuric acid method as described by Dubois (1956) was used. In this protocol 0.05ml of 80% phenol is added to 2ml of the polysaccharide solution to be tested, rapidly followed by 5ml of 95% sulphuric acid. This mixture is allowed to react for 20 minutes and then an absorbance measurement is taken at 480nm. Comparing the absorbance measurement to that of known standards will give the concentration of the polysaccharide solution being tested.

To assess alcohol content in the final product, a standard wine and spirit hydrometer was used. 70ml of the sample was poured into a graduated cylinder into which the hydrometer was placed and the alcohol content was directly read. To measure pH, a pH meter was used following the recommended procedure.

RESULTS AND DISCUSSION

For this experiment it was theorized that fermentation with a mushroom stipe extract as the liquid medium would increase the rate of fermentation and the final total ethanol content as compared to a control with no extract. As shown in Figure 3, the presence of the stipe extract did allow the fermentation to reach a higher alcohol content at a faster rate.

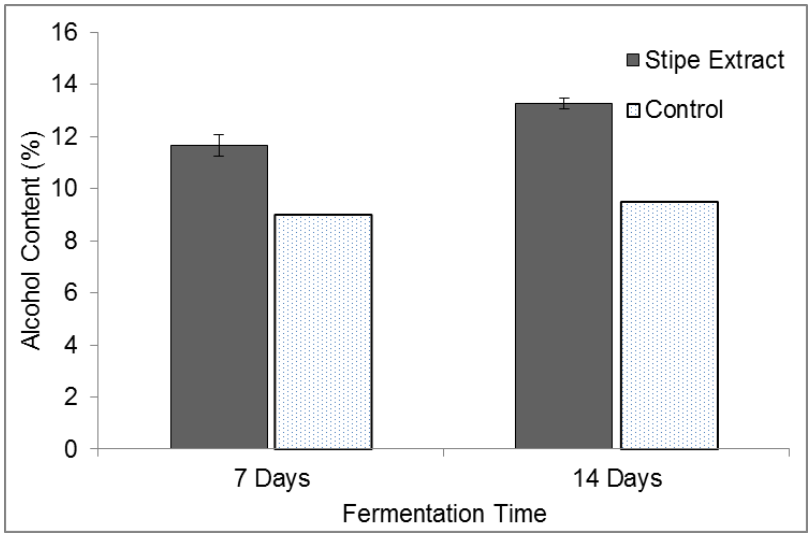

Figure 3. Alcohol content at 7 and 14 days fermentation time with and without the stipe extract

At 7 days fermentation time the alcohol content of the fermentation containing the stipe extract was 11.7% whereas the control with no extract was only 9%. This shows that the presence of the stipe extract accelerated the fermentation, leading to a higher alcohol content. Furthermore, at 14 days fermentation time the stipe extract had an alcohol content of 13.3%, showing a significant increase, while the control was only 9.5%. The presence of the stipe extract did allow for a much higher alcohol content at the end of the fermentation.

Based on a stoichiometric balance of the metabolism of glucose into ethanol and carbon dioxide, the maximum obtainable ethanol concentration is 13.9%. The fermentation with the mushroom extract was able to come much closer to the theoretical maximum as compared to the control. These results are confirmed by Lin (2010), who received a final ethanol concentration of 13.7% after 14 days when also using a mushroom stipe extract as the fermentation medium. Sucrose that was used as the carbon source in both this experiment and by Lin (2010) is composed on one molecule of glucose and 1 molecule of fructose. While *S. cerevisiae* is glucophilic, preferring glucose for metabolism, yet it is still able to ferment fructose and should be able to fully utilize the carbon source provided (Berthels 2004).

The pH of the solution can also give an indication to the growth of the yeast. Many organic acids such as citric acid, malic acid, and acetic acid are produced by yeast as minor metabolic products (Whiting 1975). The results of the pH tests are shown below in Figure 4. While the pH was higher at both time points for the fermentation with the stipe extract, indicating higher metabolism and more metabolic products, the difference is very minor.

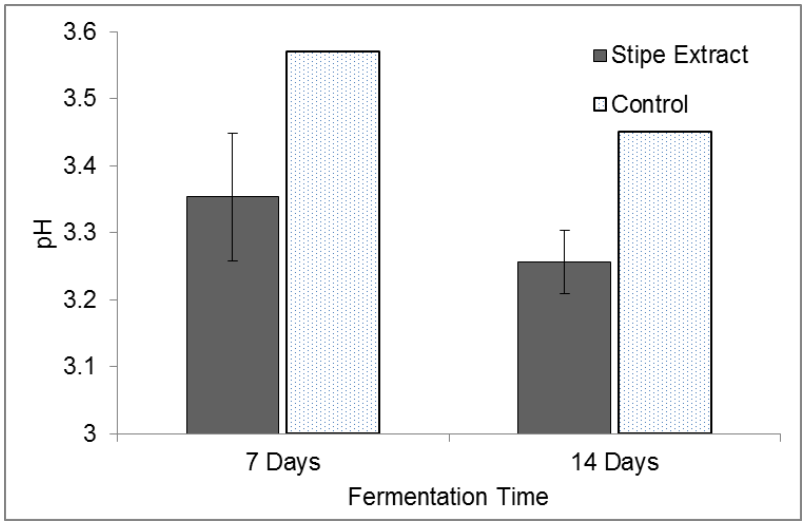

Figure 4. pH at 7 and 14 days fermentation time with and without the stipe extract

It is theorized that the reason for the increased fermentation rate is the many nutrients present in the stipe extract. The work of Lin (2010) focused on yeast assimilable nitrogen (YAN), which is required for protein synthesis and cell growth. They found that a mushroom stipe extract similar to the one replicated in this experiment had a concentration 287mg/L YAN, which is well above the 140mg/L required for optimal growth (Bely 1990). Based on the BCA assay performed on the stipe extract, the protein concentration of the stipe extract used in this experiment was approximately 726mg/L. Given that nitrogen is approximately 16% of protein (Fennema 1990) this gives a nitrogen content of 116.2mg/L. While this is significantly less than the results achieved by Lin (2010), it is still very close to the optimal nitrogen content for growth and does explain the enhanced growth rate seen in the experiment. Free nitrogen in the form of ammonia and other salts will also slightly raise the YAN content.

Another potential reason for the enhanced growth are the beta-glucans present in the mushroom stipes. Beta glucans are a primary component of chitin, the structural polysaccharide found in mushrooms (Vetter 2006) and are also an important structural component in yeast cell walls (Lesage 2006). Based on the results of the polysaccharide test performed on the mushroom stipe extract, it contained 870mg/L of polysaccharides. Knowing that the stipes contain negligible amounts of simple sugars (Vetter 2006), it can be assumed that the polysaccharides in the stipe extract are mainly complex, and potentially containing beta glucans. While no source could be found proving that environmental glucans are assimilable for yeast cell wall construction, is has been theoretically suggested (Latge 2005).

Other nutrients which could potentially explain the increased growth rate are phosphorus, biotin, and niacin, which promote the optimal growth of yeast (Boer 2010) and are all found in mushroom stipes in relatively high concentrations (Poongkodi 2015).

CONCULSION

Based on similar work done by Lin *et al.* (2010), this experiment severed to investigate if mushroom stipes could be used as a supplement to increase the rate of fermentation by *S. cerevisiae*. This would give a value-added use to mushroom stipes, which are currently regarded as waste, while also having implications in the biofuel and alcoholic beverage industries. It was successfully demonstrated that the presence of a mushroom stipe extract increased the rate of fermentation of *S. cerevisiae* leading to a higher final alcohol content. In summary, it has been demonstrate that mushroom stipe extract is able to effectively enhance the fermentation capabilities of *S. cerevisiae*, potentially by providing key nutrients required to optimize growth and metabolism.

In order to better improve the experimental results and repeatability, some considerations must be made in regards to limitations that were faced and what might be required to move forward with the experiment. One of the most immediate and easiest to identify limitations was the lack of multiple result time points. In this experiment it was only possible to test at the 7 and 14 day points due to external limitations placed upon the team. In order to unequivocally show the effects of the mushroom nutrients on the growing yeast, more robust data points need to be created. It would be highly beneficial to test at days 4, 7, 10, 14, and 21 to show the short and long term changes occurring in the solution. Finally, an important limitation was the method of measurement of alcohol content. A hydrometer was used to measure the alcohol content, and it does this by means of measuring specific gravity (relative densities) of a solution. Certain assumptions are made in the usage of a hydrometer to measure the amount of alcohol present in the solution. One of the problems present is that density is affected by the temperature of the solution, therefore the measurement of each trial must be done under the exact same temperature conditions when using a hydrometer. This presents a two-fold source of error, the first being the reading of the temperature and possible perturbations between readings, and also having to eye out the level of the hydrometer, much like a thermometer. In moving forward with this work, time and equipment availability must be prudently analyzed in order to present a more thorough examination of the hypothesis and results obtained.

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