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Evaluation of Alkaline Peroxide Pretreatment for Enhancing Reducing Sugar Yield and Lignin Removal in Wheat Straw and Spent Mushroom Substrate and its Impact on Alcohol Yield

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Abstract: ***This study investigates the effectiveness of alkaline peroxide pretreatment in enhancing reducing sugar yield and lignin removal from two lignocellulosic biomasses: wheat straw (WS) and spent mushroom substrate (SMS) derived from oyster mushroom cultivation. Pretreatment was conducted to improve the enzymatic hydrolysis efficiency and subsequent alcohol production potential. Both substrates were processed to a uniform particle size (0.82mm) and subjected to alkaline peroxide treatment. Reducing sugar concentrations were quantified using the DNS assay, while lignin content was determined via the acetyl bromide spectrophotometric method. Results revealed a significant increase in sugar yields post-pretreatment, with glucose levels rising to 127% in wheat straw and 137% in spent mushroom substrate, and xylose increasing to 115% and 128%, respectively. Concurrently, lignin content decreased markedly dropping to 53% in wheat straw and 62% in spent mushroom substrate demonstrating the efficacy of pretreatment in delignification. These enhancements suggest improved substrate accessibility and fermentation potential, indicating that alkaline peroxide pretreatment is a promising strategy for boosting bioethanol yields from agricultural waste biomass.***

Keywords: wheat straw; spent mushroom substrate; lignocellulose; oyster mushroom

1. Introduction

Oyster mushrooms (*Pleurotus* sp.) belonging to Class Basidiomycetes and Family Agaricaceae are popular among mushroom cultivators, as they can grow in a wide range of temperatures, on various lignocellulosic substrates such as rice straw, wheat straw and saw dust. Oyster mushrooms rank second among the important cultivated mushrooms in the world and account for over 80 % of the world's mushroom production **(Imran *et al.* 2011).** Associated with mushroom production is the generation of spent mushroom substrate that needs heat treatment before being removed from the growing chamber. Disposal of spent mushroom substrate being expensive, some mushroom growers discard the contaminated spent mushroom substrate far from the farm **(Rinker, 2002).** Without proper treatment, contaminated spent mushroom substrate can cause environmental problems including ground water contamination and nuisance as mushroom production is increasing so is the spent mushroom substrate generation which calls for management of this so-called waste. Conversely, recycling of spent mushroom substrate can increase sustainability and also help farm economy. The agro-residues used for mushroom cultivation consist mainly of cellulose (the major component of all plant materials) and hemicellulose (the second most important component of lignocellulosic biomass) that can be converted to ethanol after suitable processing. *Pleurotus*, which has several species such as *P. sajorcaju*, *P. ostreatus, P. florida* and *P. eous*, belong to the class Basidiomycetes. *Pleurotus* mushrooms are edible with excellent flavour and taste **(Somashekar, et al., 2010).** They have nutritional as well as medicinal properties **(Mahmood, et al., 2011)**. They are low in calories, fats, sodium, carbohydrates and cholesterol, while being rich in proteins, minerals, vitamins and fibers **(Gupta, et al., 2011).**

In lignocellulosic plant cell walls, cellulose is surrounded by and linked to hemicellulose and lignin, posing an enormous challenge for enzymes to directly convert it to monosaccharides (i.e., glucose). These physical obstacles are primarily responsible for the recalcitrance of cell walls and low cellulose accessibility, which is believed to be primarily caused by lignin and lignin-carbohydrate complexes **(McCann and Carpita, 2015)**. To overcome this recalcitrance, a pretreatment step is required to solubilize lignin and hemicellulose, break down the structural complexities of lignocellulose biomass polymers, and facilitate subsequent hydrolysis **(Kumar and Sharma, 2017)**. In addition to the solubilization of lignin and hemicellulose, pretreatment is aimed at producing cellulose with a high degree of purity and reduced crystallinity **(Feleke et al., 2023)**. Green pretreatment involves all the environmentally friendly and sustainable methods used in the separation of the components of lignocellulose biomass feedstocks for subsequent processes into biofuel or bioproducts and addresses challenges associated with the traditional pretreatment process by using milder and more sustainable approaches **(Clark, 2019)**

Lignocellulosic biomass is usually composed of agriculture residues (rice straw, corn straw, wheat straw, risk husk, sugarcane bagasse, cotton straw, and other plant residues), forest residues (wood), industrial residues (pulp and paper processing waste), and energy crops (switchgrass) **(Akhtar et al., 2016; Chen et al., 2017; Kumar and Sharma, 2017; Hassan et al., 2018)**. Generally, straw biomass is one of the agriculture residues, which is abundant, inexpensive, clean, safe, renewable, and sustainable, and can alleviate the

contradiction in applications between energy and food, which serves as the best selection to replace conventional fossil energy resources **(Steinbach et al., 2017)**. Most of the straw biomass could be transformed into numerous forms of high-value chemicals, which can reduce the environmental issues, and facilitate the sustainable development of economics and society **(Kim et al., 2012)**. Lignocellulosic biomass is principally composed of cellulose, hemicellulose and lignin in which fermentable sugars are achieved by hydrolysis of sugar components **(Tian et al., 2018)**.

Wheat straw is a typical lignocellulosic biomass that mainly comprises cellulose, hemicellulose, and lignin. Cellulose and hemicellulose could be hydrolysed into monomeric sugars such as glucose, xylose, and arabinose, which could then be converted to biofuels such as bioethanol and methane **(Jiang et al., 2016; Lopez-Linares et al., 2015; Du et al., 2016)**. However, because the chemical components and physical structure of lignocellulosic biomass could protect cellulose from degradation, the bioconversion of these materials has remained challenging **(Akimkulova et al., 20176).** Typically, lignin and hemicellulose in the lignocellulosic materials need to be removed before the enzymatic hydrolysis of cellulose **(Pei et al., 2016).** Various pretreatment approaches have been proposed and applied, including physical, chemical, and biological methods **(Song et al., 2014).** Alkali, acid, steam explosion, and hot water pretreatments have been extensively performed in related fields **(Cai et al., 2016; Liu et al., 2016; Kim et al., 2008).** Wheat straw is an abundant and cheap lignocellulosic residue, which makes it possible to be applied to fermentable sugar production at a large scale. Response surface methodology (RSM) is a statistical modelling technique that can establish a multivariate equation based on quantitative data in the designed experiments **(Hu et al., 2018; Saha and Ghosh, 2014).**

Spent substrate, the residual material of mushroom cultivation, causes disposal problems for cultivators. Currently the spent substrate of different mushrooms is used mainly for composting. Edible mushrooms of *Pleurotus sp.* can grow on a wide range of lignocellulosic substrates. *Pleurotus eous* was grown on paddy straw and the spent substrate was used for the production of ethanol. Lignocellulosic biomass cannot be saccharified by enzymes to high yield of ethanol without pretreatment. The root cause for the recalcitrance of lignocellulosic biomass such as paddy straw is the presence of lignin and hemicelluloses on the surface of cellulose. They form a barrier and prevent cellulase from accessing the cellulose in the substrate **(Koshy and Nambisan, 2011).** Mushroom cultivation, a considerable amount of spent substrate remains as residual material. SS needs heat treatment before being removed from the growing chamber. But being expensive, some mushroom growers discard the contaminated spent substrate far from the farm **(Rinker, et al., 2010).** Without proper treatment, contaminated spent substrate can cause re-contamination. Conversely, recycling of spent substrate can increase sustainability and also help farm economy. Several studies have shown the potential use of the spent substrate in purification of water and soil, cultivation of other mushroom species, cultivation of vegetables, biological control of pests, vermiculture, as well as its use as animal feed and as a source of degradative enzymes **(Castro, et al., 2008).** Alkaline pretreatment is a very popular and cost-effective lignocellulose biomass pretreatment process involving the use of an alkaline reagent for the dissolution of lignin and hemicellulose in biomass **(Kim et al., 2016)**. The dissolution of lignin and hemicellulose is due to the breakdown of the intermolecular ester bonds between lignin and hemicelluloses **(Razali et al., 2022)**. The type of alkaline, concentration, reaction time, temperature, and choice of process intensification methods are critical factors influencing the alkaline extraction of cellulose from lignocellulose biomass **(Kim et al., 2016)**. The alkalis commonly used for the removal of lignin from biomass include sodium hydroxide **(Sayakulu and Soloi, 2022)**

Enzymatic hydrolysis of the pretreated lignocellulosic substrates for fermentable sugar production, cellulases and xylanases are always used. Cellulose can be hydrolysed via the synergy actions of β-1,4-endoglucanases (EC 3.2.1.4); β-1,4-exoglucanases, which are also named cellobiohydrolases (EC 3.2.1.91); and β-D-glucosidases (EC 3.2.1.21). Hemicellulose (Xylan) can be hydrolysed through the coordination of Endo xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). No matter what type of enzymes are used, enzymatic activities can be influenced by factors including temperature, pH, enzyme loading and biomass loading. Therefore, it is necessary to optimize the enzymatic hydrolysis of the pretreated substrates to enhance sugar yields and enzymatic hydrolysis efficiency. In addition, the adoption of inexpensive enzymes and substrates during enzymatic hydrolysis can reduce the input cost of hydrolysis **(Zhang and Wu, 2023).** Worldwide increasing energy demand and the accompanying environmental degradation brought about by conventional energy sources, it is essential that we look inwards for alternative sources of energy and chemicals **(Abonagye et al., 2017)**. Therefore, the conversion of plant biomass into useful products such as biofuels and biochemicals has been increasing due to improved economic feasibility of conversion processes **(Blotkamp et al., 1978)**. As an important resource, lignocellulose biomass provides sustainability, and its economically friendly **(Ishizawa et al., 2007)**. Currently, we can categorize biofuels into first and second generations. The first-generation biofuels are obtained from sugars, starch, and plant oils. These are derived from feedstocks such as cereals, sugarcane, sugar beet, grapes, soya, palm, and sunflower **(Ghose, 1987)**. The second-generation biofuels are from lignocelluloses such as softwoods, hardwoods, municipal wastes, and agricultural wastes **(Sun and Cheng, 2002)**. In present study we focused on pretreatment process analysis with wheat straw and spent mushroom substrate waste to production bioethanol in form of reducing sugar.

2. Materials and Methods

In this study, we evaluated the effect of alkaline peroxide pretreatment to yield reducing sugar and degrade the lignin. lignocellulosic biomass used for production of bioethanol includes wheat straw (WS) and spent mushroom substrate

(SMS) of Oyster Mushroom, both were processed using distinct methods.

2.1 Wheat Straw and Spent Mushroom Substrate Processing

Wheat straw was sourced from local agricultural fields and carefully cleaned to remove contaminants such as soil and plant debris. It was then ground to a uniform particle size of 0.82mm using a laboratory mixer. This specific particle size was deliberately selected to ensure uniformity across samples, making subsequent experimental procedures more manageable. The fibrous structure and particle distribution of the wheat straw were carefully documented to gain insights into its behaviour within a composite material matrix. Spent mushroom substrate was obtained as a byproduct from controlled laboratory mushroom cultivation experiments conducted in this study. To ensure consistency in substrate composition and quality, oyster mushrooms were grown using wheat straw as the primary substrate.

2.2 Oyster Mushroom Cultivation process

The cultivation of oyster mushrooms using wheat straw as a substrate is a systematic process designed to maximize yield while minimizing contamination. The first step involves chopping the wheat straw into smaller pieces, which increases the surface area available for mycelium colonization, thereby enhancing the efficiency of the cultivation process. After chopping, the straw is treated with a chemical sterilizing agent, Bavistin (a fungicide), which helps to eliminate harmful fungi and other unwanted microbes. Bavistin is effective in preventing contamination during the early stages of mushroom cultivation. Following this chemical treatment, the straw is autoclaved for approximately 25 minutes at 121 °C to ensure complete sterilization. Autoclaving not only removes contaminants but also softens the straw, making it easier for the mushroom mycelium to penetrate and colonize **(Mahari et al., 2020)**.

Once sterilization is complete, the straw is allowed to cool and is then drained to remove excess moisture. In a sterile environment, the straw is inoculated with oyster mushroom spawn. The spawn is mixed evenly throughout the substrate to promote uniform colonization. The inoculated straw is packed into perforated plastic bags or containers, which allow for proper aeration an essential factor for the growth of the mycelium. The bags are then incubated in a dark, warm room, typically at 20-24°C with high humidity. This incubation period lasts for about 2-3 weeks, during which the mycelium spreads through the straw, fully colonizing it. Once colonization is complete, the bags are moved to a fruiting chamber where conditions are modified lower temperatures of 15-20°C, increased light exposure, and high humidity levels of 85-95% to trigger mushroom fruiting. Within days to weeks, oyster mushrooms begin to develop and can be harvested when they reach the desired size. This method ensures sustainable and efficient mushroom production, with careful control over environmental conditions to maximize output and quality **(Girmay et al., 2016)**.

2.3 Reducing sugar estimation using DNS method

The DNS (3,5-Dinitrosalicylic Acid) assay was employed to measure reducing sugars in both pretreated and non-pretreated biomass hydrolysates. Initially, the hydrolysate samples were diluted 20 times with distilled deionized water. A calibration curve was created utilizing glucose standards made from a 20mM stock solution, with final concentrations varying from 0 to 5mM attained via serial dilutions after which 150µl of DNS reagent was added and mixed well. The tubes were subsequently immersed in a boiling water bath for 5 minutes, enabling the reducing sugars to interact with the DNS reagent and create a coloured complex. Once sample cooled to room temperature, the absorbance was assessed at 540nm using a spectrophotometer, with a blank sample (0mM glucose standard with DNS reagent) utilized for calibration **(Tishler et al., 2015).**

2.4 Lignin quantification using the acetyl bromide spectrophotometric method

Lignin quantification was performed using the Acetyl Bromide Spectrophotometric Method, a highly sensitive UV-Vis technique specifically suited for peroxide-pretreated lignocellulosic biomass. Initially, 5–10mg of dried, pretreated biomass (wheat straw and spent mushroom substrate) was carefully weighed and transferred into vial. To ensure complete lignin solubilization, 1mL of acetyl bromide (25% in glacial acetic acid) was added to the sample, and the reaction mixture was incubated at 50°C for 30minutes. After incubation, the reaction mixture was allowed to cool to room temperature, and the volume was adjusted using acetic acid to achieve a standardized dilution. The absorbance of the resulting solution was measured at 280nm using a UV-Vis spectrophotometer, with appropriate blank and control samples to ensure accuracy **(Barnes and Anderson, 2017).**

3. Results and Discussion

3.1 Effect of Alkaline Peroxide Pretreatment on reducing sugar and lignin

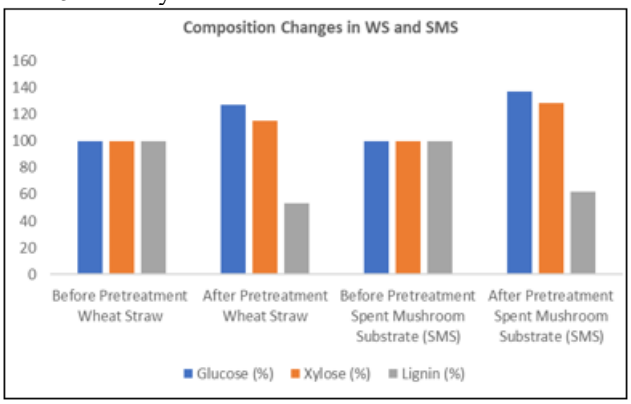
The lignocellulosic biomass wheat straw and spent mushroom substrate, contains glucose, xylose, and lignin, and the pretreatment process significantly impacts these components. Prior to pretreatment, the levels of these components were considered 100%. Following pretreatment, glucose content increased to 127% in wheat straw and 137% in Spent Mushroom Substrate, reflecting improved sugar availability due to the breakdown of complex carbohydrates.

Table 1: Alkaline Peroxide Pretreatment on Glucose, Xylose, and Lignin Levels in Wheat Straw and Spent Mushroom Substrate

Substrate	Glucose (%)	Xylose (%)	Lignin (%)
Before Pretreatment Wheat Straw	100	100	100
After Pretreatment Wheat Straw	127	115	53
Before Pretreatment Spent Mushroom Substrate	100	100	100
After Pretreatment Spent Mushroom Substrate	137	128	62

Similarly, xylose content rose to 115% in wheat straw and 128% in Spent Mushroom Substrate, indicating enhanced hemicellulose hydrolysis. In contrast, all percentage data showed by table-1 and graph-1, lignin content decreased markedly, dropping to 57% in wheat straw and 58% in Spent Mushroom Substrate, demonstrating the pretreatment's effectiveness in lignin removal. These changes emphasize the role of pretreatment in increasing fermentable sugar release while reducing lignin, making the biomass more suitable for biofuel production.

Gould (1984 and 1985) reported that Alkaline hydrogen peroxide pretreatment of 2% biomass with 0.5g H_2O_2/g biomass was essentially complete at 8h. At a biomass loading of approximately 8.6% (w/v) and an H_2O_2 loading of approximately 0.25 g/g biomass, **Saha and Cotta (2006)** found that alkaline hydrogen peroxide pretreatment was essentially complete at 6h. However, neither of these studies examined the effect of extended residence time at low H_2O_2 loadings. In fact, extending the residence time from 24h to 48h at a low H_2O_2 loading (0.125 g/g biomass), with no pH adjustment, caused an increase in Glucose yields in response to Accellerase 1000 from 46% to 58%. With a four-component enzyme mixture optimized specifically for this pretreatment condition, Glucose yields could be increased further to 66.3%. Accellerase 1000 alone, pH adjustment or extended residence time caused a similar enhancement of monomeric Glucose yields (from 46% up to 58% to 59%), but when using an optimized commercial cocktail, pH adjustment caused a bigger enhancement than increased pretreatment time (from 66% to 74%). In regard to biomass loading during pretreatment, the earlier results indicated that 10% loading was superior to 2%, 6%, or 8%. Therefore, even higher loadings were tested. At loadings of 15% or 20%, with subsequent digestion with Accellerase 1000 alone, sugar yields improved by 4% to 8% for Glucose and 3% to 5% for Xylose.



Graph 1: Changes in wheat straw and spent mushroom substrates composition Before and After Pretreatment

Before pretreatment, wheat straw was subjected to compositional analysis on dry basis weight (w/w) and the contents of cellulose (45%), hemicelluloses (21.30%) and lignin (19%) were determined. This data provides the information about the effect of 2.5% sodium hydroxide (alkaline solution) at various steaming times (30, 60, 90 and 120min) on structural changes that take place in wheat straw **(Irfan et al. 2010).** The result of earlier study showed that maximum cellulose and delignification was achieved at 2.5% sodium hydroxide **(Nadeem et al. 2013).** Initially, the wheat straw was kept at 121°C steams under pressure for 30min, where 67% cellulose, 17.30% hemicelluloses and 63.5% delignification were achieved. In the same way, the substrate was pretreated for 60min the cellulose (77.51%) and delignification (72.5%) increased but reduction was observed in hemicelluloses (14.6%) as compared with 30min steam under pressure. Moreover, the steaming time was further increased where the substrate was treated for 90min, resulting in the maximum cellulose content of 83% and delignification of 81%, whereas the hemicelluloses decreased further to 10.50%. The reduction in hemicelluloses and highest value of cellulose indicate the removal of hemicelluloses from wheat straw **(Rahnama et al. 2013).** Furthermore, when the substrate was treated for 120min, a reduction in cellulose contents (76%) was observed. These results expressed that treatment at 121°C for 120min has some harsh effect on lignocellulosic biomass as compared with 90min which may degrade the lignin, hemicelluloses and also solubilised cellulose contents.

4. Conclusion

The application of alkaline peroxide pretreatment significantly enhanced the fermentable sugar yield and reduced lignin content in both wheat straw and spent mushroom substrate. Spent mushroom substrate exhibited slightly higher improvements in sugar release, likely due to partial degradation during prior fungal treatment. The pretreatment elevated glucose and xylose concentrations while effectively reducing lignin to nearly half of its original content. These findings underline the potential of this pretreatment method to improve substrate digestibility and optimize bioethanol production. Overall, this study supports the utilization of agro-industrial residues like wheat straw and spent mushroom substrate as viable, sustainable feedstocks for biofuel generation following appropriate chemical pretreatment.

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