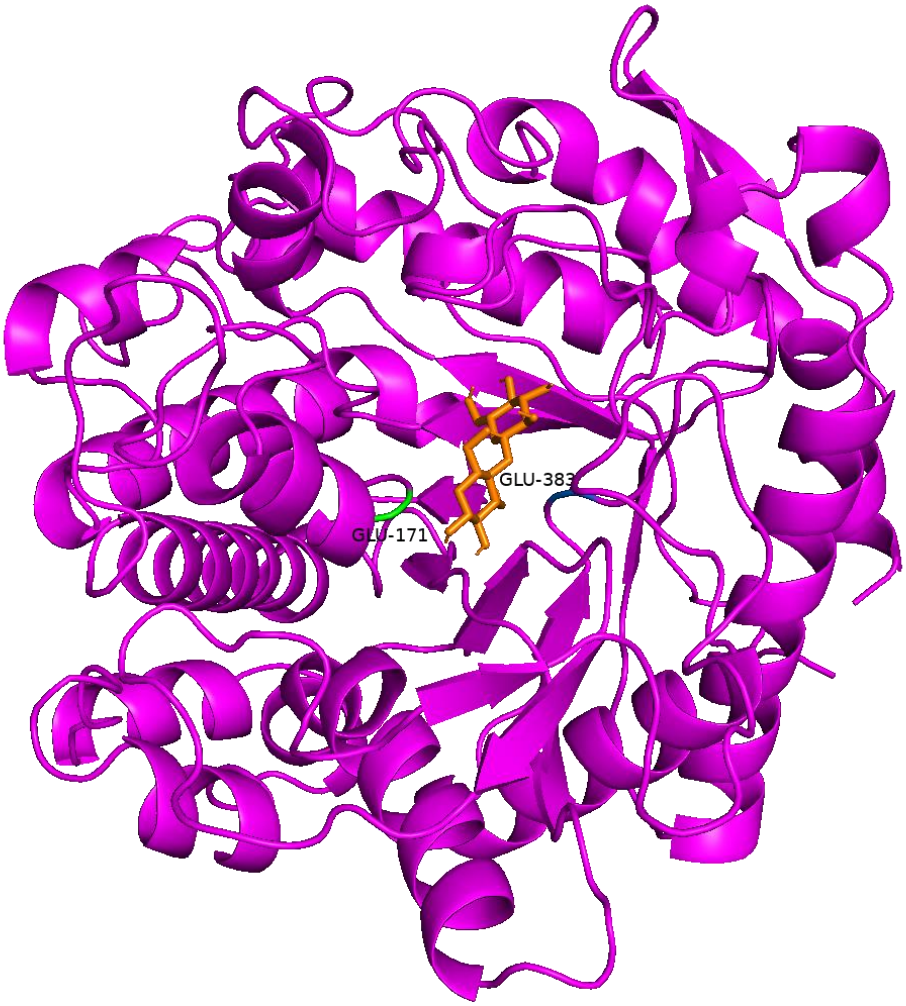

Acidophilic fungal beta-glucosidase(s) for biorefinery applications

Bachelor Thesis in Chemical Engineering

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Contents

List of Abbreviations

BGL:	Beta-glucosidase
BLAST:	Basic Local Alignment Search Tool
CAZy:	Carbohydrate-Active enZYmes
CBB:	Coomassie Brilliant Blue
dBCAN:	Database for Carbohydrate-Active enZYmes
FBM:	Fungal Basal Medium
GH:	Glycoside Hydrolase
HPLC-RI:	High-Performance Liquid Chromatography with Refractive Index detection
kDa:	Kilodalton
pNP:	p-nitrophenol
pNPG:	p-nitrophenyl β-D-glucopyranoside
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SignalP:	Signal Peptide prediction tool
Talaromyces-358:	Talaromyces sp. ASS 358-9
w/v:	Weight/Volume

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Acknowledgments

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Abstract

Lignocellulose is a promising substrate for the production of chemicals and materials. In traditional biorefineries, it is first pretreated to disrupt its structure, thereby yielding acidic fractions. Therefore, enzymes from acid-tolerant organisms would be advantageous for their bioconversion. In this study, we have investigated beta-glucosidases (BGLs) from the fungi *Talaromyces sp1. ASS 358-9 (Talaromyces-358)*, which thrives in highly acidic conditions and can utilize inhibitor-rich liquor from spruce pretreatment for growth and enzyme production. Since BGLs are crucial for biomass saccharification, we are studying the properties of differentially expressed *Talaromyces-358* BGLs, aiming to find robust enzymes for biorefinery use that can withstand harsh environments like high temperatures and low pH levels. Culturing *Talaromyces-358* in various liquid media revealed glucose as the most favorable substrate, significantly enhancing BGL activity over 15 days, while spruce liquor, though initially inhibitory, eventually supported significant enzyme production. The BGLs exhibited peak activity at 80°C and maintained considerable activity at 90°C, with optimal pH ranging from 4 to 6, demonstrating adaptability to harsh conditions. Enzyme activity was measured using substrates such as glucose, glucose-spruce, spruce, xylan, and xylan-spruce, with temperature measurements taken from 30°C to 90°C at pH 4, and pH measurements taken from 2 to 9 at 50°C. However, there was little to no BGL activity observed at neutral and basic pH levels.

Keywords

Beta-glucosidase (BGL), *Talaromyces sp. ASS 358-9 (Talaromyces-358),* Enzyme activity, Thermal stability, pH dependence, Substrate specificity, Lignocellulose bioconversion, Biorefineries

Introduction

In the global search for alternative energy sources, lignocellulosic biomass is a crucial renewable and sustainable resource. Because of its wide availability and renewable nature, it is essential to the transition away from finite fossil fuels, which is necessary to maintain the long-term viability of our planet [1]. However, because of its complex lignin-hemicellulose-cellulose matrix, utilizing lignocellulosic biomass in conventional biorefineries creates a significant obstacle [2]. Because of its structural complexity, it requires extensive pretreatment procedures in order to optimize its ability for bioconversion into useful compounds. One of the most important processes in the creation of sustainable liquid fuels is the enzymatic hydrolysis of the biomass, which converts the cellulose into fermentable sugars like glucose [1][2][3].

Nonetheless, the heterogeneous composition of lignocellulosic biomass, which includes cellulose, hemicelluloses, lignin, and extractives-hinders the effectiveness of enzymatic hydrolysis by generating barriers for successful bioconversion [2][4]. Pretreatment, therefore, becomes an essential preparatory process that aims to increase cellulose's accessibility by decreasing its crystallinity, increasing its surface area, and eliminating compounds that block it, such as lignin and hemicelluloses [2][4][3]. Although pretreatment techniques exhibit potential for industrial application, problems with cost-effectiveness, waste management, and product yield optimization still exist [2]. Additionally, the selected steam explosion (STEX) pretreatment method generates acidic fractions that frequently have the potential to interfere with the activity of conventional bioconversion enzymes [5]. This occurs because the STEX process breaks down hemicelluloses and lignin, releasing acetic acid and other acidic compounds, which can lower the pH and inhibit enzyme activity [5].

The cooperative activity of BGLs, cellobiohydrolases, and endo-1,4-beta-glucanases is necessary for the hydrolysis of cellulose. Although cellulose polymers are broken down by endo-glucanases and cellobiohydrolases, BGLs play a vital role in reducing product inhibition because they turn cellobiose into glucose [6]. However, efficient biomass hydrolysis is hampered by challenges associated with BGLs, such as their vulnerability to product inhibition by glucose and thermal instability. These drawbacks underline the necessity of novel strategies that enhance enzymatic efficiency and overcome challenges in the conversion of lignocellulosic biomass [6].

The optimization of lignocellulosic biomass conversion requires an integrated approach that includes the discovery of new enzymes as well as the improvement of current ones. Finding new BGLs by screening, using proteomic or genomic techniques, can lead to the discovery of enzymes with greater durability and activity. In addition, specific alterations to improve enzyme function are made possible by advances in enzyme engineering, such as directed evolution and rational design [2]. Through the utilization of advanced biotechnological instruments and the wide range of fungal species available, scientists can stimulate novel approaches to producing enzymes and establish environmentally friendly and economically viable biorefinery procedures.

Thesis Objectives

The primary objectives of this thesis are:

1. **Identify Optimal Carbon Source for BGL Secretion:** Systematically evaluate various carbon sources to determine the most effective one for maximizing the secretion of beta-glucosidase (BGL) enzymes during cultivation of *Talaromyces sp. ASS 358-9 (Talaromyces-358)* under controlled laboratory conditions.

2. **Characterize and Select Robust BGL Enzymes for Biorefinery Applications:** Investigate, characterize, and select beta-glucosidase (BGL) enzymes from *Talaromyces sp. ASS 358-9 (Talaromyces-358)* based on their activity, thermal stability, and pH tolerance to identify enzymes with the highest potential for efficient lignocellulosic biomass hydrolysis in industrial biorefinery settings.

Background

Pretreatment in Biorefineries

Conventional pretreatment methods are essential for breaking down the complex structure of lignocellulosic biomass, which is composed of cellulose, hemicellulose, and lignin. These methods can be classified into several categories, each employing different mechanisms to achieve the desired disruption and fractionation of biomass.

The principal function of biomass pretreatment is to increase plant polysaccharide accessibility to biocatalysts for fermentation into sugars that can be fermented [3][5]. A successful pretreatment technique must, however, also take into account a number of secondary objectives, such as decreasing the production of inhibitory compounds, preventing sugar losses, enabling the recovery of valuable byproducts like lignin, and optimizing energy usage [3]. A number of parameters, including the specified level of conversion and the initial material's physical and chemical composition and structural qualities, influence the choice of pretreatment strategy [3][5].

The four categories of pretreatment techniques are physical, chemical, physico-chemical, and biological. Physical pretreatments like milling and grinding reduce biomass particle size, increasing surface area and making it more suitable for chemical or biological treatments to improve enzymatic hydrolysis [7]. Chemical pretreatments, such as acid and alkali treatments, enhance enzymatic hydrolysis by breaking down hemicellulose and lignin, making cellulose more accessible [3][5][8]. Another effective pretreatment is utilizing oxidizing agents, organic solvents, and ionic liquids to also improve hydrolysis [3][5]. Physico-chemical methods like liquid-hot water pretreatment and steam explosion further simplify biomass structure for further processing [7]. Biological methods use enzymes and microorganisms to break down complex polysaccharides into fermentable sugars, increasing conversion efficiency. Enzymatic treatments target cellulose and hemicellulose, while microbial degradation employs bacteria and fungi to degrade lignin and hemicellulose, offering a more environmentally friendly approach [3][5].

Enzyme hydrolysis challenges

Apart from sugar derivatives such as mono- and oligosaccharides, biomass pretreatment produces a variety of water-soluble and insoluble inhibitory compounds. The levels of these inhibitors are closely linked to both the structural characteristics of the biomass and the specific pretreatment techniques applied. Given the complex composition of lignocellulosic biomass, extensive pretreatment is necessary to facilitate the enzymatic hydrolysis. The pretreatment enhances the accessibility of the cellulose, as the removal of hemicellulose exposes the cellulose fibers for enzymatic hydrolysis [9]. Hemicellulose shielding the cellulose influence the overall efficiency of enzymatic hydrolysis.

Throughout this pretreatment process, the biomass undergoes degradation, yielding a range of inhibitory compounds. These include phenolic substances such as coniferyl alcohol/aldehyde, vanillin, and ferulic acid derived from lignin, alongside non-phenolic acids like benzoic acid and cinnamic acid. Furthermore, acids like acetate, formate, and levulinate, as well as aldehydes like furfural and its hydroxymethylated derivative, are produced from the hemicellulose portion of lignocellulose. Phenolic acids originating from lignin, such as tannic acid and gallic acid, notably impede the activity of BGL from *Trichoderma reesei* [2][9]. Therefore, enzymes from acid-tolerant organisms would be advantageous for their bioconversion and would be of interest for further investigation [5].

Acidophilic Microorganisms and Their Enzymes

Extremely acidic environments, defined by pH levels below 3, represent an interesting but rather uncommon phenomenon compared to mildly acidic conditions found in soils and certain aquatic habitats [10]. These extreme conditions, whether natural or anthropogenic in origin, largely result from the accumulation of sulfuric acid, a consequence of sulfur's abundance in various oxidation states on Earth [10]. Primary sources of extreme acidity include volcanic and geothermal regions, where oxidation processes generate pH values close to zero, as observed in locales like Lake Kawah Idjen in Indonesia and the Copahue volcano region in Argentina [10]. Additionally, acidic environments have also been detected in mining locations. For example, the Richmond Mine in California has recorded negative pH values as low as -3.6 due to high concentrations of sulfuric acid and hydronium ions. These extreme conditions arise from extensive evaporation and the formation of 'acid-generating salts'. Extreme acidity also occurs as a result of interactions involving sulfuric compounds in settings such as extensive cave systems or sewage networks [10].

Numerous eukaryotic branches feature acidophiles, or organisms that have adapted to live in extremely acidic conditions. These include algae, fungus, amoebas, and flagellates [10]. Certain physiological characteristics of these organisms allow them to survive in extremely acidic environments, such as the capacity to sustain notable pH gradients and the use of various techniques for maintaining pH homeostasis and repairing damage prevention strategies, which include DNA repair and synthesis of acid stable proteins [10]. Acidophiles face additional challenges beyond extreme acidity, such as high concentrations of transition metals and metalloids, high osmotic pressures, and fluctuating temperatures, which they manage through various mechanisms [10].

The unique enzymatic properties and participation of fungi from harsh environments, especially acidophilic fungi, make them highly desirable for biotechnological applications [11]. Acidophilic fungi produce enzymes that remain active in highly acidic environments, making them beneficial for processes like ethanol production from lignocellulosic materials [11]. Because of their capacity to flourish in acidic environments, they also reduce microbial contamination, which is advantageous for several biotechnological processes [11].

The industrial interest in extremophiles is driven by their ability to thrive in extreme environments, which often translates to enzymes with high stability and activity under harsh conditions, such as elevated temperatures, extreme pH, and high salinity [12]. Extremozymes, enzymes produced by extremophiles, have gained prominence in biotechnological applications due to their capacity to catalyze reactions under such severe conditions [12]. As a result of the increasing demand and profitability of extremophiles, the global market for industrial enzymes and extremozymes has grown significantly. A recent study stated that the market for unusual enzymes, such as extremozymes, was estimated at $4.0 billion globally in 2018 [12]. Therefore, extremozymes are excellent candidates for investigation, providing new avenues for researchers to meet the growing global demand 12].

Role of Beta-glucosidases (BGLs) in Biomass Saccharification

Beta-glucosidases (BGLs) are crucial in the saccharification of biomass, particularly in the enzymatic hydrolysis of cellulose. These enzymes catalyze the final step in the breakdown of cellulose by converting cellobiose and other short oligosaccharides into glucose [13]. This function is essential because cellobiose, if not removed, inhibits the activity of cellobiohydrolases and endoglucanases, the other enzymes involved in the initial steps of cellulose breakdown [14]. The accumulation of cellobiose creates a bottleneck in the hydrolysis process, reducing the overall efficiency of biomass conversion to fermentable sugars [13][14]. Thus, the presence and activity of efficient BGLs are vital for achieving high yields of glucose.

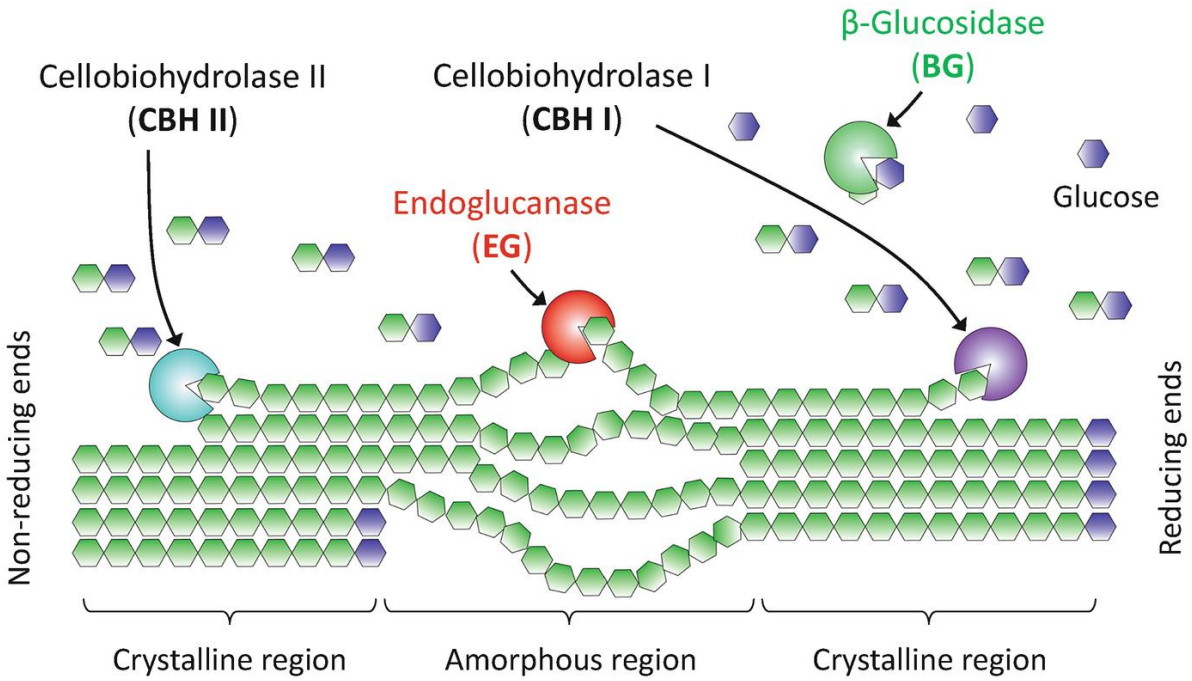

Figure 1: *Breakdown of cellulose through enzymatic hydrolysis [15].*

The three primary types of enzymes are involved in the fundamental process of hydrolyzing cellulose: beta-glucosidases, exo-1,4-beta-glucanases (also known as cellobiohydrolases), and endo-1,4-beta-glucanases. Together, these enzymes work in coordination to gradually and systematically convert cellulose polymers into glucose [14]. Endo-glucanases mainly target the internal 1,4-beta-linkages in the amorphous areas of cellulose. The cellulose polymer is subsequently treated by exo-1,4-beta-glucanases from the free ends, which results in the processive release of cellobiose as a product. Ultimately, cellobiose is then hydrolyzed into glucose by beta-glucosidases [14].

In the industrial context, several factors limit the efficiency of biomass hydrolysis, including product inhibition by glucose, thermal inactivation of enzymes, and the high cost of enzyme production [14]. BGLs are particularly sensitive to inhibition by their product, glucose, which significantly hampers their activity over time. Therefore, identifying BGLs that maintain high activity despite glucose accumulation is a priority [14]. Additionally, enzymes must be stable at the elevated temperatures and varied pH levels typical of industrial processes.

Chapter 1: BGL Production

1.1 Introduction

The production of beta-glucosidase (BGL) enzymes plays a pivotal role in the bioconversion of lignocellulosic biomass into fermentable sugars, which are crucial for various biotechnological applications, including biofuel production. The fungi *Talaromyces 358-9* was sampled from mountain soil in Mai Son, Son La, Vietnam. This chapter outlines the methods and processes involved in the cultivation of the fungi for BGL production. It details the experimental setup for culturing the fungus in different liquid media, the preparation of substrates, and the conditions necessary for optimal fungal growth and enzyme secretion. The chapter also describes the procedures for conducting the BGL assay using pNPG, establishing standard curves, and analyzing BGL activity over a 15-day period. Furthermore, it covers the enzymatic hydrolysis of Avicel and provides a comprehensive analysis of glucose release using High-Performance Liquid Chromatography with Refractive Index detection (HPLC-RI).

1.2 Culturing of the fungus *Talaromyces-358* in liquid media

The liquid growth medium, known as Fungal Basal Medium (FBM), is carefully formulated with containing 4.0 g/L potassium phosphate, 13.60 g/L ammonium sulfate, 0.80 g/L calcium chloride, 0.60 g/L Epsom salt, 0.10 g/L peptone, 0.10 g/L yeast extract, and 0.21 g/L Tween-80. Additionally, an FBM Trace Elements solution (1,000x) is prepared, containing 10.0 g/L iron sulfate, 3.20 g/L manganese sulfate, 2.80 g/L zinc sulfate, 4.0 g/L cobalt chloride, and 3.50 g/L copper sulfate.

In the culturing process, the fungi are cultured in 100 mL flasks containing the media solution. The differences between the flasks were the substrates used, which provided the source of carbon and energy for the fungi. 5 different substrate combinations were tested: glucose, glucose-spruce, spruce, xylan, and xylan-spruce. Each liquid media variant is divided into three separate flasks, each labeled with the substrate name and a unique number, resulting in a total of 15 liquid media configurations.

The spruce sample was specifically a spruce liquor, that were analysed to determine its individual components. The analysis revealed the presence of several monosaccharides with the following concentrations, 1.82 g/L arabinose, 4.00 g/L galactose, 57.00 g/L glucose, 7.20 g/L xylose, and 24.50 g/L mannose.

To prepare the FBM, various substrates are incorporated at a 0.5% (w/v) concentration and sterilized. After sterilization, the medium's pH is carefully adjusted to approximately pH 3 by adding a few drops of filter-sterilized 10% (v/v) sulfuric acid solution. Subsequently, the medium is inoculated with a 1% volume of a spore suspension containing 0.03 mL of spore stock solution, with a spore count of 1 x 10^7 spores/mL. This results in a total flask volume of 100 ml and a final spore concentration of 1 x 10^5 spores/mL.

Maintaining optimal conditions is crucial for successful fungal growth. Therefore, the inoculated FBM is kept at a temperature of 30°C with agitation at 150 rpm. These controlled

conditions provide the necessary environment for the *Talaromyces-358* fungus to thrive and develop in the liquid media.

1.3 Preparation of buffers and solutions for the pNPG assay

To conduct the BGL assay using pNPG, a series of buffers with varying pH values ranging from 2 to 9 were prepared. Each buffer had a concentration of 0.05 M, created by adjusting the pH of the different components mixed with 30 mL of Milli-Q water with either hydrochloric acid or sodium hydroxide, and then adjusting the final volume to 50 mL with Milli-Q water.

Different components were used to prepare the buffers based on the desired pH range. For Sodium Citrate buffers with pH values from 2 to 5, sodium citrate dihydrate and citric acid were utilized. Sodium Phosphate buffers with pH values from 6 to 7 were prepared using sodium phosphate dibasic heptahydrate and sodium phosphate monohydrate. For Tris buffers with pH values of 8 and 9, Trizma base was used.

To prepare the necessary solutions for the enzymatic assays, three different solutions were prepared. The 1M sodium carbonate solution was made by combining 300 mL of Milli-Q water with 31.797 grams of sodium carbonate, ensuring a homogeneous mixture. The 2mM pNPG solution was prepared by combining 8.8 mg of pNPG with a pH 4 buffer, adjusting the total volume to 14.6 mL to achieve the desired concentration. For the 0.2 mM p-nitrophenol (pNP) solution, a 20 mM stock solution was initially prepared by combining 5.4 mL of pNP with 1.94 mL of pH 4 buffer. To obtain the 0.2 mM solution, 0.2 mL of the stock solution was withdrawn and mixed with 1.8 mL of pH 4 buffer, ensuring accurate measurement and achieving the desired concentration for use in the enzymatic assays.

1.4 Procedures for establishing a pNP standard graph and conducting the BGL assay.

For the enzymatic assays, two key procedures were conducted: the establishment of a pNP standard graph and the BGL assay.

To construct the pNP standard graph, a series of pNP standard solutions were prepared, covering a range of known concentrations from 0.002 µmol to 0.018 µmol. These solutions were subjected to incubation at 50°C for 10 minutes, followed by the addition of 100 µl of 1M sodium carbonate to halt the reaction. The absorbance at 405 nm was then measured using a microplate reader. By plotting the absorbance values against the known concentrations of pNP, a standard curve was generated, facilitating the determination of pNP concentrations in subsequent experiments.

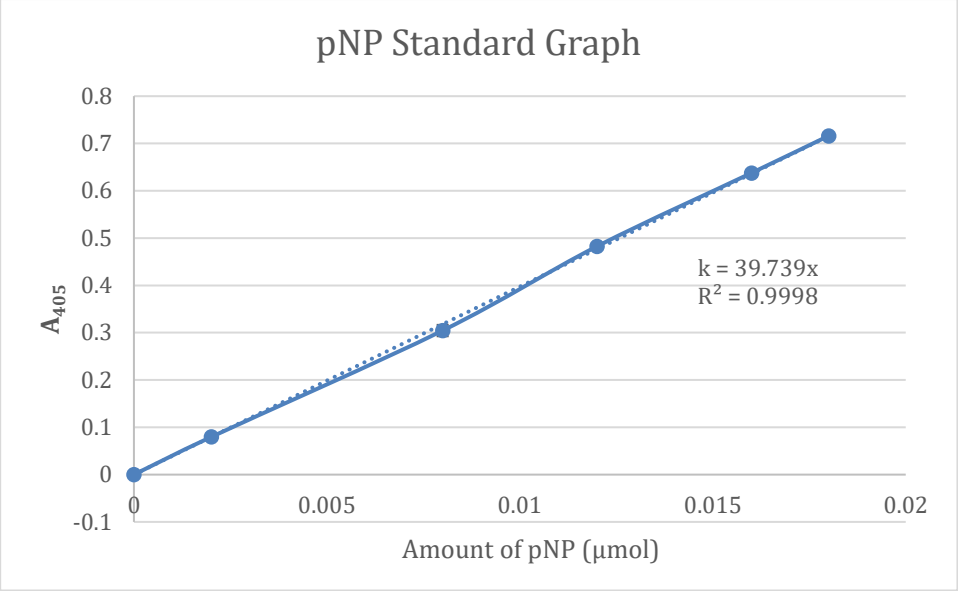

Figure 2: *pNP standard graph used to determine BGL activity.*

In the BGL assay, the activity of the BGL enzyme was determined using a microplate-based assay. Enzyme test mixtures were prepared by combining the BGL enzyme with 2 mM pNPG substrate in microplate wells. These mixtures were then incubated at 50°C for 10 minutes with agitation. Substrate blank mixtures that only contained the substrate and enzyme blank that contained one half substrate and the other half enzyme were also prepared as controls. Following incubation, the reactions were terminated by adding 100 µL of 1 M sodium carbonate to each well. The absorbance at 405 nm was measured using a microplate reader. BGL activity was determined by referencing the absorbance values with the standard curve established in Figure 2.

Calculations for BGL Activity (µmol/mL/min) or (U/mL):

$$\frac{A_{405}-S_{405}-E_{405}}{k\times 0{,}05\times 10}$$

where:
A_{405} is the absorbance of the test sample.
S_{405} is the absorbance of the substrate blank.
E_{405} is the absorbance of the enzyme blank.
k is the slope of the pNP standard curve.
0.05 is the volume of the enzyme used in mL.
10 is the factor to convert the rate per minute.

1.5 Analysis of BGL activity over time using different substrates

To evaluate the performance of *Talaromyces 358-9* in producing BGL enzymes, the activity was measured over a 15-day period using various substrates. The substrates included glucose, glucose-spruce, spruce, xylan, and xylan-spruce. This analysis aimed to determine how different substrates influence BGL production and to assess the enzyme's adaptability and efficiency in different conditions. The results are depicted in the following figure, which shows the changes in BGL activity over time for each substrate.

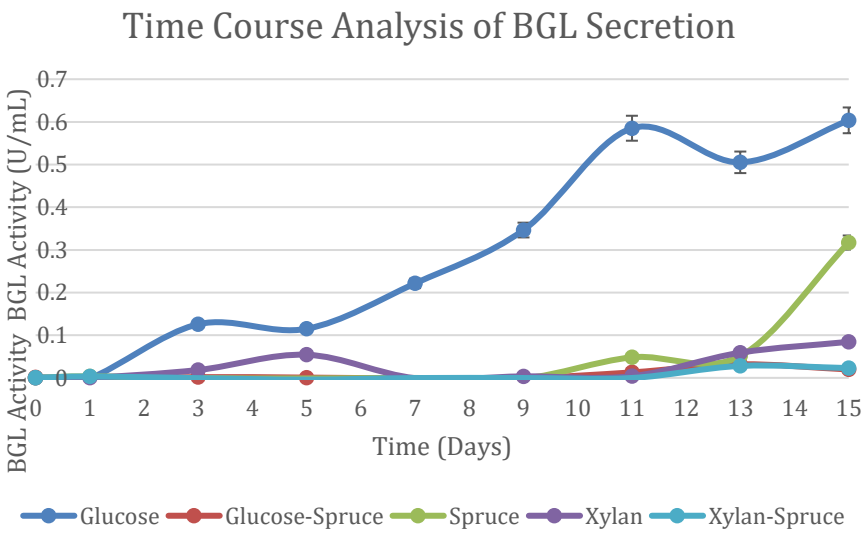

Figure 3: *Graph depicting the BGL activity of Talaromyces 358-9 measured over a 15-day period using the different substrates.*

Glucose: BGL activity showed a consistent increase from 0.00025 U/mL on day 0 to 0.604 U/mL on day 15, indicating that glucose is a favourable substrate for BGL production. The steady rise in activity suggests a strong affinity and effective enzyme production in glucose-rich environments.

Glucose-Spruce: BGL activity fluctuated, with an initial low value on day 0 (0.0012 U/mL) but eventually reaching 0.020 U/mL by day 15. These fluctuations imply that the presence of spruce introduces inhibitory compounds that initially affect enzyme production. Over time, *Talaromyces-358* appears to adapt, showing improved activity, which may be due to metabolic adjustments overcoming the spruce inhibitors.

Spruce: BGL activity varied significantly, starting near zero and staying there until day 9 when it is beginning to increase, and finally peaking at 0.32 U/mL on day 15. This significant variation and eventual peak suggest that spruce alone is a challenging substrate due to its complex and inhibitory nature. The initial low values highlight the difficulty in directly utilizing spruce, but the peak on day 15 indicates potential adaptation and optimization of enzyme production by *Talaromyces-358*.

Xylan: BGL activity increased steadily from 0.00061 U/mL on day 0 to 0.084 U/mL on day 15, with a notable spike to 0.054 U/mL on day 5. This progressive increase suggests that

Talaromyces-358 can effectively utilize xylan, with the spike potentially resulting either from the initial breakdown of xylan components, releasing sugars that further stimulate BGL production, or that the *Talaromyces-358* is a mycelium fungus that started a growth phase during that time span.

Xylan-Spruce: BGL activity showed initial fluctuations but gradually increased to 0.023 U/mL by day 15. These results indicate that while xylan-spruce is initially challenging, *Talaromyces-358* can adapt to this mixed substrate. The gradual increase in activity suggests that the organism can overcome initial inhibitors and produce enzymes efficiently in this mixed environment.

In summary, *Talaromyces-358* produced BGL to varying degrees, depending on the substrate of the culture and the length of the culture. The steady increase in BGL activity during cultivation on glucose and xylan highlights their suitability for enzyme production. *Talaromyces-358's* ability to adapt and eventually produce significant BGL activity when cultivated on spruce, which contains inhibitory compounds, indicates its potential for efficient lignocellulose bioconversion. These findings suggest that *Talaromyces-358* could be promising for biorefinery applications. Further optimization could enhance enzyme production. Additionally, strategies to mitigate inhibitors would improve its efficiency.

1.6 Testing BGL activity on cellulose

In the enzymatic hydrolysis process employing BGL, a methodical sequence of steps is followed to efficiently degrade lignocellulosic biomass. Two samples were prepared: Cellulase and Cellulase + BGL, each with a total hydrolysis volume of 5 ml. The Cellulase sample contains 0.5 g of Avicel cellulose, 0.25 ml of 1M pH 4 buffer, 0.25 ml of Tween 80, 0.13 ml of cellulase, and 3.99 ml of water. The Cellulase + BGL sample includes 0.5 g of Avicel, 0.25 ml of 1M pH 4 buffer, 0.25 ml of Tween 80, 0.096 ml of BGL from the glucose sample, 0.13 ml of cellulase, and 3.91 ml of water. The reaction mixtures are incubated at 50 degrees Celsius for 24 hours, with intermittent sampling conducted throughout the incubation period to monitor progress.

After incubation, the hydrolysate is recovered through centrifugation at 19g for 30 minutes to separate the soluble fraction from the solid biomass. The recovered samples are then filtered using 0.2-µm syringe filters to eliminate any remaining particulate matter. For analytical assessment, the filtered samples undergo HPLC-RI analysis.

Performing HPLC-RI involves precise steps using a Rezex ROA Organic Acid H+ column with 5mM sulfuric acid as the mobile phase. The column is maintained at 80°C with a flow rate of 0.8 mL/min. Samples are diluted, filtered through a 0.2 µm filter, and injected into the HPLC system after equilibrating with the mobile phase. The RI detector measures the change in refractive index as each compound elutes from the column, producing a chromatogram. The peak areas of the sample chromatogram are compared with those of known glucose standards to identify and quantify the amount of glucose in the sample.

To quantify glucose release, a calibration curve is constructed using glucose standard solutions at various concentrations. These standards are injected into the HPLC-RI system,

and their peak areas are recorded. The peak areas are then plotted against the known concentrations to generate the calibration curve. Filtered hydrolysate samples are injected into the HPLC-RI system, and the retention time and peak area of the glucose in each sample are recorded. The glucose concentration in the hydrolysate samples is calculated using the calibration curve, converting the peak area of each sample to glucose concentration based on the linear equation derived from the calibration curve.

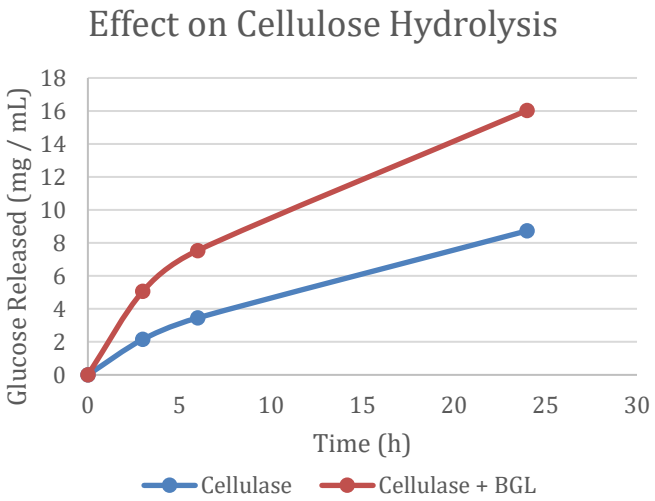

Figure 4: *Graph of the glucose released over a 24-hour period, measured using HPLC-RI.*

The analysis reveals a gradual increase in glucose release over a 24-hour period under both Cellulase and Cellulase + BGL conditions. Initially, there is no glucose release observed in either condition. However, as time progresses, both conditions show a steady rise in glucose release, indicating enzymatic breakdown of cellulose.

Under enzymatic hydrolysis applying Celluclast, glucose release increases steadily over time. Meanwhile, the Cellulase + BGL condition exhibits a more pronounced increase in glucose release, indicating enhanced enzymatic activity and more efficient cellulose breakdown. This difference underscores the impact of the BGL on cellulose hydrolysis efficiency, leading to accelerated glucose release.

1.7 Conclusion

The production of beta-glucosidase (BGL) enzymes by the fungus *Talaromyces-358* has been effectively demonstrated, showcasing the organism's ability to adapt and produce enzymes under various substrate conditions. The experimental procedures involved culturing the fungus, preparing substrates, and conducting enzymatic assays, providing a framework for evaluating BGL activity. Results indicate that glucose is the most favourable substrate for BGL production, with consistent increases in enzyme activity observed over a 15-day period. The experiments also highlight the potential of *Talaromyces-358* to adapt to complex substrates like spruce, suggesting its suitability for biorefinery applications. Additionally, the analysis of enzymatic hydrolysis on Avicel cellulose underscores the combined efficiency of cellulase and BGL in hydrolyzing cellulose. The enzymatic hydrolysis of Avicel showed a significant increase in glucose release over a 24-hour period, with more pronounced glucose release observed in the presence of both cellulase and BGL compared to cellulase alone.

Chapter 2: Physico-chemical properties of secreted BGLs

2.1 Introduction

Understanding the properties of beta-glucosidase (BGL) enzymes is crucial for optimizing their application in industrial processes, particularly those involving the conversion of lignocellulosic biomass into fermentable sugars. This chapter investigates the effects of temperature and pH on BGL activity, providing insights into the enzyme's stability and efficiency under varying environmental conditions. As before, the media differed by the substrates they included: glucose, glucose-spruce, spruce, xylan, and xylan-spruce. By analyzing these Physico-chemical in the different medias, the chapter aims to identify the optimal conditions for maximizing BGL activity.

2.2 Analysis of BGL activity at different temperatures

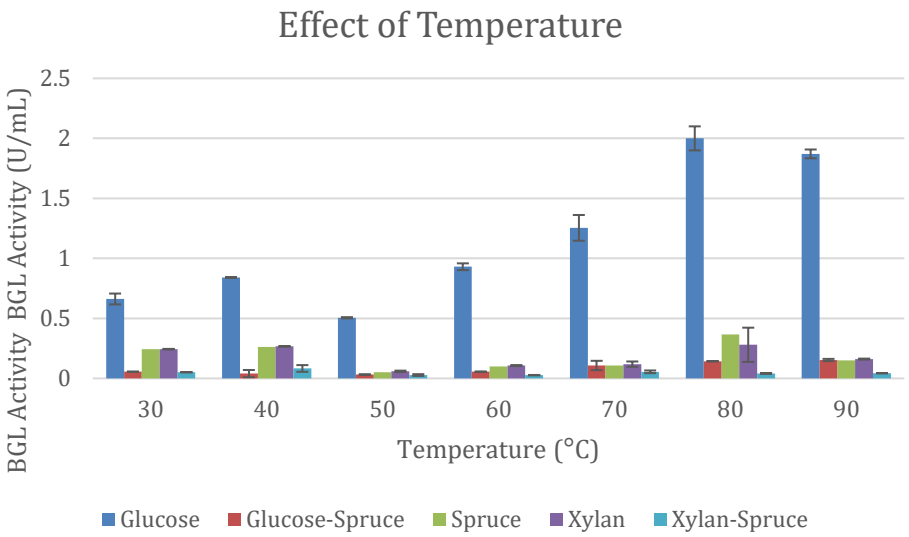

Figure 5: *Graph of the BGL activity of Talaromyces-358 measured at different incubation temperatures ranging from 30°C to 90°C using the different substrates at pH 4.*

Glucose: BGL activity increased with temperature, peaking at 80°C with 2.00 U/mL before slightly decreasing at 90°C to 1.87 U/mL. This trend indicates that *Talaromyces-358* has the highest BGL activity at elevated temperatures, with optimal activity around 80°C, suggesting the enzyme's thermal stability and efficiency at high temperatures.

Glucose-Spruce: BGL activity showed a moderate increase across temperatures, starting at 0.06 U/mL at 30°C and reaching 0.15 U/mL at 90°C. This gradual increase suggests that while the enzyme adapts to the mixed substrate, its activity is not as pronounced as in the glucose sample, indicating an effect when spruce is added to the media. However, the rising trend indicates potential for use in varied thermal conditions.

Spruce: BGL activity varied significantly, with a peak at 80°C (0.37 U/mL). The activity was lower at other temperatures, with a minimum at 50°C (0.05 U/mL). This indicates that while *Talaromyces-358* can produce BGL in the presence of spruce, its efficiency is highly temperature-dependent, with optimal activity at higher temperatures.

Xylan: BGL activity showed an increasing trend with temperature, peaking at 80°C with 0.28 U/mL. The lowest activity was observed at 50°C (0.06 U/mL). This pattern suggests that xylan is effectively utilized by *Talaromyces-358* for enzyme production at higher temperatures, demonstrating the enzyme's thermal adaptability.

Xylan-Spruce: BGL activity displayed a fluctuating trend, with the highest activity at 40°C (0.08 U/mL) and another peak at 70°C (0.06 U/mL). The activity at 80°C and 90°C was relatively lower compared to the other substrates, indicating that the mixed substrate of xylan and spruce might present additional challenges for enzyme activity, particularly at higher temperatures.

In summary, the BGL activity of *Talaromyces-358* demonstrates significant potential. This is particularly due to its robust performance across various temperatures. The highest BGL activity was observed with glucose at 80°C, reaching 2.00 U/mL, indicating the *Talaromyces-358'*s exceptional thermal stability and efficiency. Across all substrates, BGL activity generally increased with temperature, peaking between 70°C and 80°C. Notably, the enzyme maintained considerable activity even at 90°C, which underscores its functionality at higher temperatures. This characteristic is particularly advantageous for industrial processes that require high-temperature operations [16]. The varying activity across different media and temperatures suggests the possibility that *Talaromyces-358* produces different kinds of BGLs or isoforms, each adapted to specific environmental conditions. The ability of *Talaromyces-358* to produce BGL in the presence of complex and inhibitory substrates like spruce and mixed substrates like xylan-spruce further highlights its adaptability and potential for efficient lignocellulose bioconversion.

2.3 Analysis of BGL activity at different pH levels

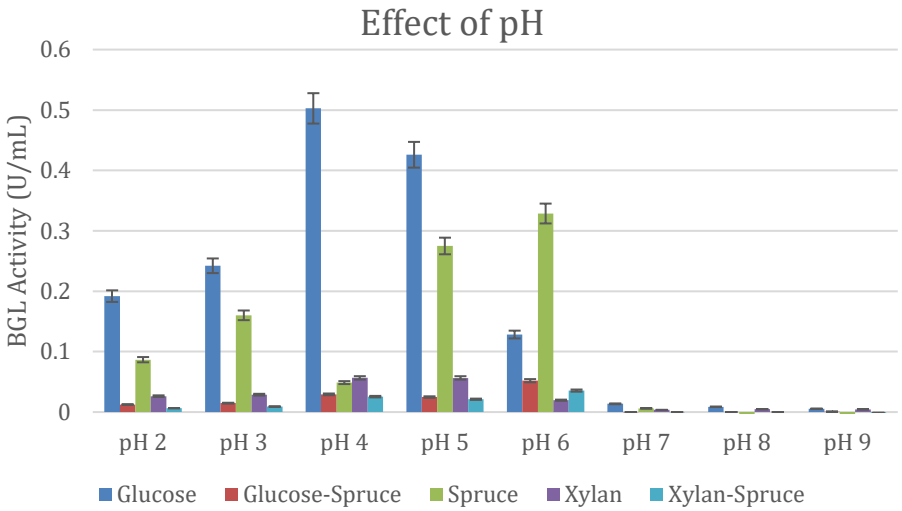

Figure 6: *Graph of the BGL activity of Talaromyces-358 measured at different incubation pH ranging pH 2 to 9 using the different substrates at a constant temperature of 50°C.*

Glucose: BGL activity was highest at pH 4, reaching 0.50 U/mL. Activity remained relatively high at pH 5 (0.43 U/mL) but decreased significantly at pH 6 (0.13 U/mL) and almost ceased at pH levels 7, 8, and 9. This indicates that the enzyme is most effective in slightly acidic conditions and loses activity in neutral and basic environments.

Glucose-Spruce: BGL activity peaked at pH 6 (0.050 U/mL) and was moderately high at pH 4 (0.030 U/mL). However, activity was low at pH levels 2 and 3 and dropped to almost zero at pH 7 and 8. This suggests that the enzyme can tolerate a range of pH levels, with optimal activity in mildly acidic conditions.

Spruce: BGL activity showed a peak at pH 6 (0.33 U/mL) and significant activity at pH 5 (0.27 U/mL) and pH 3 (0.16 U/mL). Activity was lower at pH 4 (0.05 U/mL) and minimal at pH levels 7, 8, and 9, indicating that the enzyme performs best in mildly to moderately acidic environments when using spruce as a substrate.

Xylan: BGL activity was highest at pH 4 (0.06 U/mL) and pH 5 (0.06 U/mL), showing optimal performance in slightly acidic conditions. Activity decreased significantly at pH 6 (0.02 U/mL) and was minimal at higher pH levels, indicating reduced enzyme efficiency in neutral and basic conditions.

Xylan-Spruce: BGL activity peaked at pH 6 (0.040 U/mL) and was moderately high at pH 4 (0.03 U/mL). Activity was lower at pH 5 (0.020 U/mL) and almost negligible at pH levels 7, 8, and 9, indicating optimal enzyme performance in mildly acidic environments.

In summary, the BGL enzyme from-358 exhibits varying activity across different pH levels depending on the substrate. Generally, BGL activity is highest in slightly acidic conditions (pH 4 to 6), with optimal performance at pH 4 for glucose (0.50 U/mL) and pH 6 for spruce (0.33 U/mL). The enzyme's activity diminishes significantly in neutral and basic conditions, indicating

that *Talaromyces-358* 's BGL enzyme is most effective in acidic environments. The differing activity levels across various pH conditions and substrates suggest the possibility that *Talaromyces-358* produces different kinds of BGLs or isoforms, each optimized for specific environmental conditions. This adaptability underscores the potential of *Talaromyces-358* for applications in environments where maintaining an acidic pH is necessary.

2.4 Conclusion

This chapter examines the effects of temperature and pH on the activity of beta-glucosidase (BGL) enzymes produced by *Talaromyces-358*. The results show that BGL activity peaks at 80°C, demonstrating high thermal stability. The enzyme also maintains substantial activity at 90°C, which is advantageous for high-temperature industrial processes. Optimal pH conditions for BGL activity vary by substrate, with the highest activity observed in slightly acidic environments (pH 4 to 6). Specifically, BGL activity peaks at pH 4 for glucose and pH 6 for spruce. This is beneficial since biorefinery pretreatment processes are often acidic. The variations in activity suggest that *Talaromyces-358* produces different BGL isoforms. Additionally, it may produce distinct enzymes depending on the substrate. These enzymes adapt to the specific environmental conditions created by each substrate. Overall, the ability of *Talaromyces-358* to produce BGL enzymes that maintain considerable activity under acidic conditions and at high temperatures underscores its potential for future applications.

Chapter 3: Identification of putative BGLs

3.1 Introduction

The application of advanced protein analysis techniques is crucial for gaining a comprehensive understanding of beta-glucosidase (BGL) enzyme expression and activity. This chapter focuses on the methodologies and results of SDS-PAGE and Native PAGE experiments, which are used to analyze protein expression profiles. Additionally, it includes a genome analysis of *Talaromyces-358* to identify potential BGL sequences. By integrating these techniques, the chapter provides a detailed view of protein expression and identifies promising BGL candidates for biotechnological applications, particularly in the field of biomass conversion.

3.2 Method for performing SDS-PAGE

To perform SDS-PAGE for the separation of proteins based on their molecular weight, the following steps were taken:

First, the sample preparation was conducted. 10 µL of 4X Laemmli Sample Buffer was mixed with 30 µL of protein sample. If the sample colour did not turn blue, 1 µL of 1M Tris base was added to adjust the pH. After ensuring proper mixing, the samples were heated at 95°C for 5 minutes to denature the proteins.

For the electrophoresis setup, the Mini-PROTEAN Tetra Cell system was used. A Mini-PROTEAN TGX Stain-Free gel was inserted into the cell, and the tank was filled with 1X SDS Running Buffer. 40 µL of the prepared sample mixture was loaded into each well, including the marker (Precision Plus Protein Unstained Standards) for reference. To prevent the last well from migrating away in the gel, 5 µL of sample buffer was added to the well next to the last well. The gel was run at 120V until the dye front reached the reference mark.

After the run, the gels were activated and viewed under UV light using the ChemiDoc Imaging System. For more sensitive analysis, the gels were stained overnight in QC Colloidal Coomassie Stain. After staining, the gels were destained with water and viewed under visible light using the ChemiDoc Imaging System.

3.3 Results from SDS-PAGE experiment

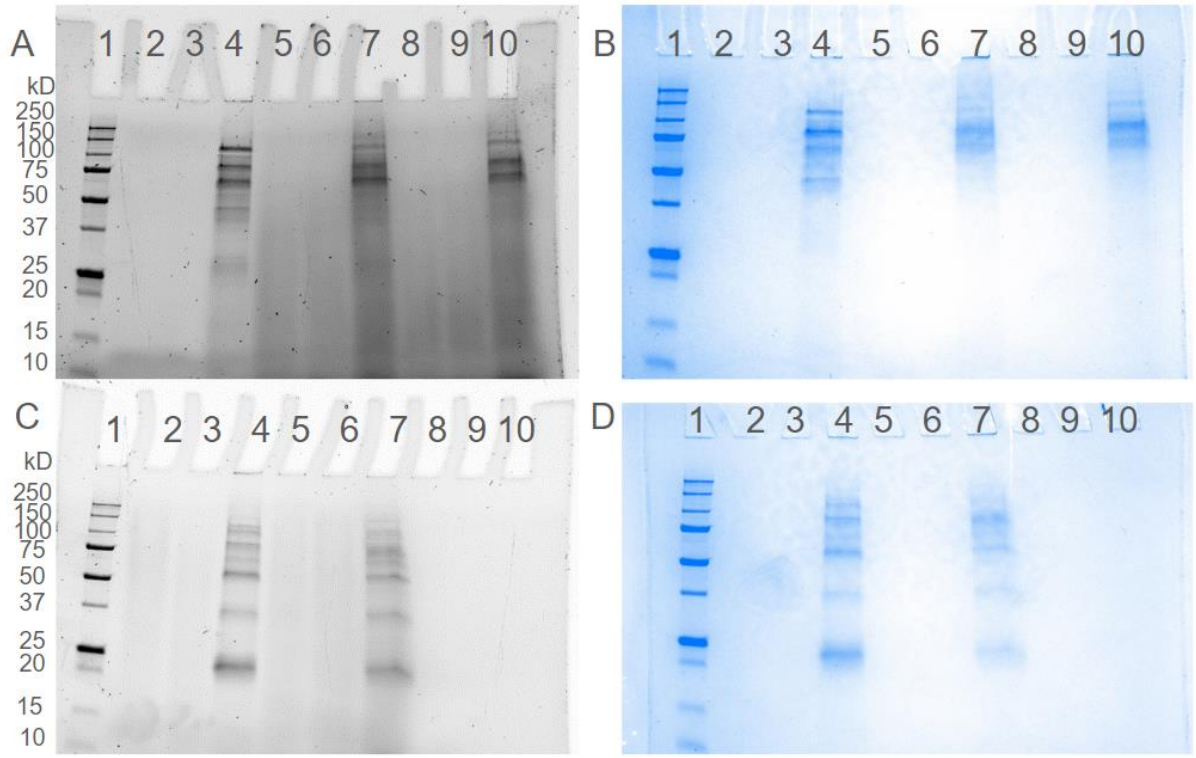

Figure 7: *SDS-PAGE separation patterns.*

The figure depicts SDS-PAGE separation patterns using Mini-PROTEAN TGX Stain-Free gels. Each gel includes the Precision Plus Protein Unstained Standards marker in well 1, with matching protein profile visible on the left side of panels A and C. Panels A and C are unstained, while panels B and D are stained with Coomassie Brilliant Blue (CBB). In panels A and B, wells 2, 3 and 4 contain Glucose samples from day 0, 1, and 15, respectively. Wells 5, 6 and 7 contain Glucose-Spruce samples from the same time points, and wells 8, 9 and 10 contain Spruce samples also from the same time points. Similarly, in panels C and D, wells 2, 3 and 4 hold Xylan samples from day 0, 1, and 15, respectively. Wells 5, 6 and 7 contain Xylan-Spruce samples from these same time points.

Panels A and B: Glucose, Glucose-Spruce, and Spruce Samples
The protein bands observed in these wells suggest a stable protein expression profile over time. Notably, there are no visible bands in the day 0 or day 1 wells, indicating minimal protein presence or expression at these early time points and no interference from media components.

In the Glucose Samples (Wells 2-4), the protein bands in these wells are primarily around 50 to 100 kDa, with a faint band at approximately 25 kDa. This indicates that the major proteins expressed in glucose media are within this molecular weight range. Meanwhile the Glucose-Spruce Samples, these samples show a similar protein profile to the glucose samples, with prominent bands between 50 kDa and 100 kDa. However, the band at 25 kDa is present but less intense compared to the glucose samples, suggesting a slight reduction in the expression of lower molecular weight proteins when spruce is added to the glucose medium. In the Spruce Samples, the protein profile is similar to that of the glucose-spruce samples, with bands ranging from 50 kDa to 100 kDa. However, these bands are less pronounced, indicating a

lower overall protein expression or stability in the spruce-only medium compared to the glucose-spruce combination.

Panels C and D: Xylan and Xylan-Spruce Samples
Similar to Panels A and B, the protein bands in these wells indicate a stable protein expression profile over time. Again, there are no noticeable bands in the day 0 or day 1 wells, suggesting minimal protein presence at these initial time points.

In the xylan samples solutions (Wells 2-4), the protein profile in these samples shows significant bands ranging from 25 kDa to 150 kDa. However, these bands are generally less pronounced than those observed in the glucose and glucose-spruce samples, indicating a different protein expression profile or lower overall protein levels in the xylan medium. In the Xylan-Spruce Samples (Wells 5-7), these samples exhibit a similar protein profile to the xylan samples, with bands from 25 kDa to 150 kDa. The primary difference is that the bands around 150 kDa are less pronounced, while the band around 75 kDa is more intense, suggesting a shift in protein expression or stability when spruce is added to the xylan medium.

3.4 BGL activity analysis using Native Page

For Native PAGE, which allows the separation of proteins without denaturing them, the following steps were performed:

Protein samples were mixed with the appropriate amount of Native Sample Buffer. If necessary, the pH was adjusted by adding 1 µL of 1M Tris base to ensure the colour turned blue. For Glucose, Spruce, and Xylan-Spruce, 3 µL of Native Sample Buffer was mixed with 6 µL of sample. For Glucose-Spruce and Xylan, 5 µL of Native Sample Buffer was mixed with 10 µL of sample.

The Mini-PROTEAN Tetra Cell was set up for electrophoresis, and a Mini-PROTEAN TGX Stain-Free gel was inserted into the system. The tank was filled with 1X Native Running Buffer. The prepared sample mixtures were loaded into each well, including the NativeMark Unstained Protein Standard as a marker. To prevent the last well from migrating away in the gel, 5 µL of Native Sample Buffer was added to the well next to the last well. The gel was run at 120V until the dye front reached the reference mark.

For visualization, the gel was washed in distilled water and incubated for 10 minutes at 50°C in a buffer containing 10 mM pNPG at pH 4. After incubation, the gel was destained with water and viewed under UV light using the ChemiDoc Imaging System. Additionally, the gel was examined on a lightboard to map out if a yellow dot from the pNPG existed, which would indicate the presence of active BGL.

3.5 Results from Native PAGE experiment

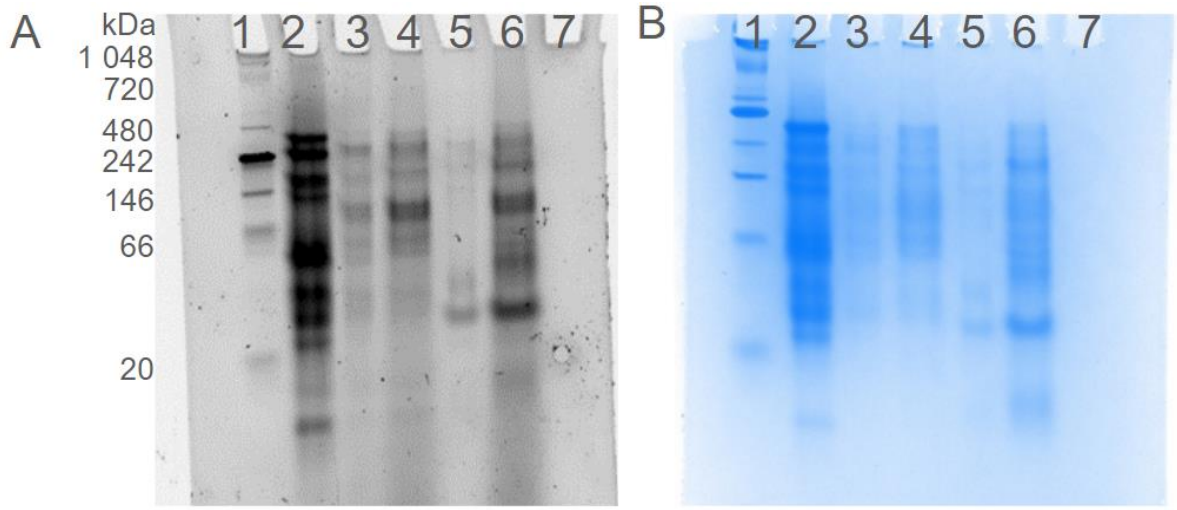

Figure 8: *Native Page separation patterns.*

The figure depicts Native Page separation patterns on Mini-PROTEAN TGX Stain-Free gels. Both panels A and B contain the NativeMark Unstained Protein Standard marker in well 1 with matching protein ladder on the left side of the panel. All samples are taken from day 15. Panel A is unstained, while panel B is stained with Coomassie Brilliant Blue (CBB). The samples in the wells are as follows: well 2 contains Glucose, well 3 contains Glucose-Spruce, well 4 contains Spruce, well 5 contains Xylan, and well 6 contains Xylan-Spruce.

By comparing panels A and B, it is clear that the staining confirms the presence of distinct bands at the expected molecular weights, validating the robust protein expression observed. The intensity of these bands indicates a strong protein expression across different samples. The most noticeable different bands are seen in the Glucose (Well 2) and Xylan (Well 5) samples.

The Glucose sample exhibits the most prominent and widespread protein bands, ranging from 20 kDa to nearly 480 kDa, with an additional band below 20 kDa. This suggests a diverse protein expression profile in the glucose medium. In contrast, the Xylan sample shows the fewest major bands, with a prominent band around the 20 kDa mark and several minor bands extending up to 480 kDa.

The Xylan-Spruce sample (Well 6) is similar to the Glucose sample but with less intense bands, indicating slightly lower protein expression. The Glucose-Spruce (Well 3) and Spruce (Well 4) samples exhibit comparable profiles, with the Glucose-Spruce sample showing slightly more pronounced bands. This suggests that the addition of glucose to spruce moderately enhances protein expression.

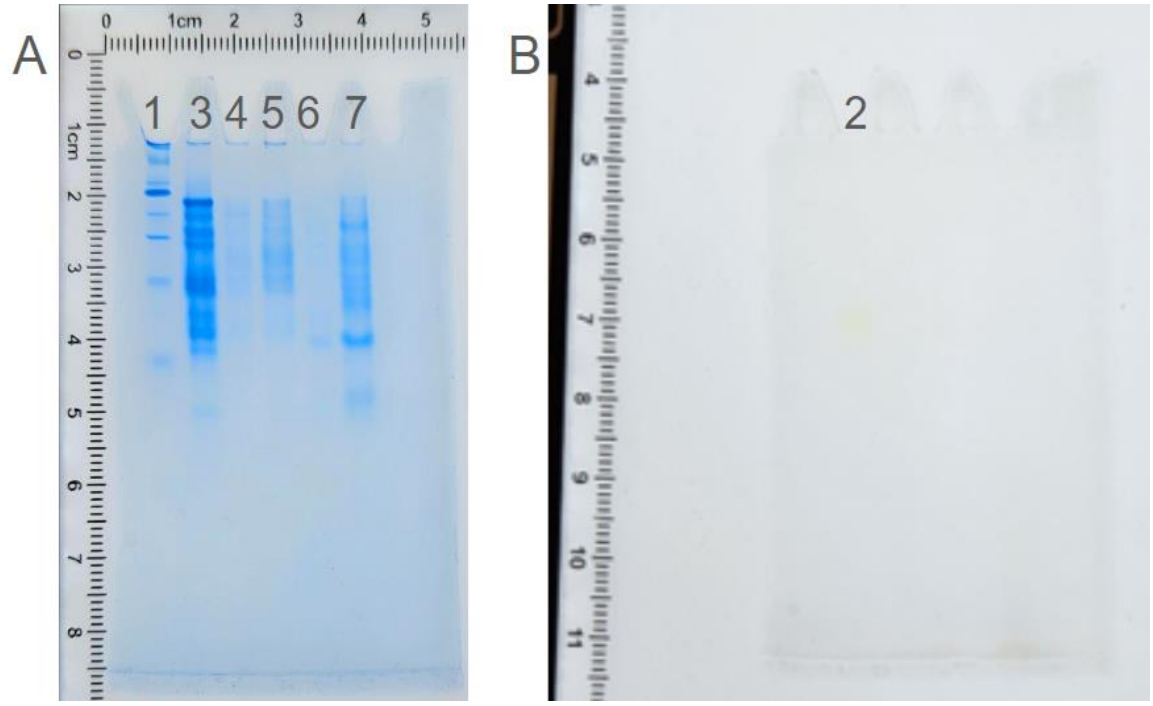

Figure 9: *Native Page separations on lightboard.*

The figure depicts Native Page separation patterns on Mini-PROTEAN TGX Stain-Free gels. Panel A has the same Gel from figure 6, but taken on a Light Board with marked measurements. The samples in the wells are as follows: well 1 NativeMark Unstained Protein Standard marker, well 3 contains Glucose, well 4 contains Glucose-Spruce, well 5 contains Spruce, well 6 contains Xylan, and well 7 contains Xylan-Spruce. Panel B is from a similarly made gel, but unstained and incubated with 10mM pNPG, pH4 and at 50 degrees for 10 minutes. Well 2 contains Glucose.

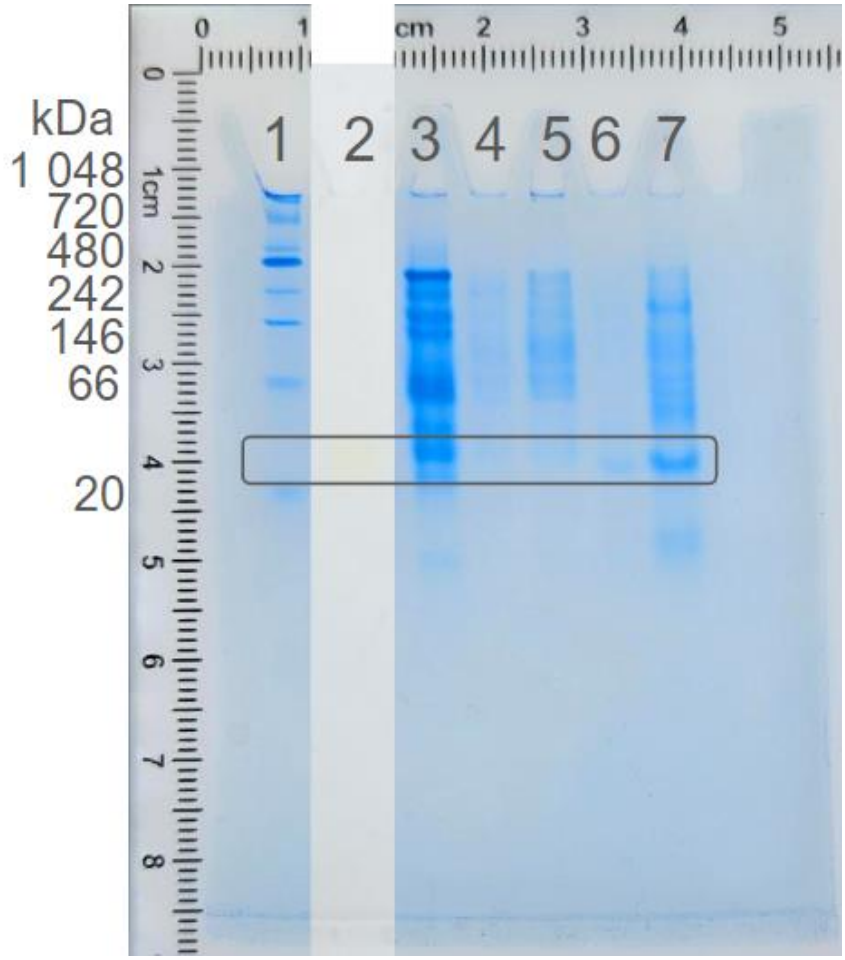

Figure 10: *An overlap of Figure 9 with a box highlighting the yellow dot and the corresponding band of the same size.*

As noted before, pNPG is a chromogenic substrate used for detecting BLG activity, which hydrolyzes pNPG to release para-nitrophenol, producing a yellow colour. The appearance of a yellow dot around the 30 kDa mark in the Glucose sample after incubation with pNPG, suggests the presence of active BLG, indicating that *Talaromyces-358* expresses active BLG in the glucose medium.

Within the marked box, it is important to note that all other media also show protein bands at approximately the same level as the yellow band, albeit to varying degrees. The Xylan-Spruce sample (Well 7) has the most noticeable band at this level. This similarity in band position across different media suggests that the protein responsible for BGL activity is expressed in multiple substrates, though the intensity and possibly the activity level of this enzyme vary.

Despite the expectation that multiple enzymes contribute to BGL activity, only a single prominent yellow dot appears in the glucose well. This could be due to several factors. One possibility is that the enzyme responsible for the yellow dot is expressed at significantly higher levels in the glucose media compared to other substrates. This high expression level could overshadow the presence of other BGL enzymes. Another reason might be that the enzyme in the glucose well is more active or stable under the assay conditions, leading to a more detectable signal. Additionally, specific inhibitors or varying environmental conditions, such as

temperature and pH levels, in other media might reduce the visibility of other BGL enzymes, preventing the appearance of yellow dots in those wells.

3.6 Genome analysis for identifying potential BGL sequences

Table 1: *Overview of the sequence screening process*

Step	Number of Sequences
Predicted proteins based on genome sequencing	12,974
Sequences in CAZy families	974
Sequences in GH family	302
Potential BGL sequences	27
BGL sequences with SignalP	11

3.6.1 Sequence Filtering and CAZy Family Identification

A genome analysis of *Talaromyces-358* was utilized to identify enzymes relevant to lignocellulose bioconversion, particularly focusing on BGLs. Initially, a total of 12,974 sequences (obtained after genome sequencing and protein prediction) were screened using dBCAN, a tool for identifying carbohydrate-active enzyme (CAZy) families [17]. Through this process, 974 sequences were identified as belonging to CAZy families, indicating their potential role in carbohydrate metabolism.

3.6.2 Identification of GH Family Sequences

Among the 974 CAZy-associated sequences, 302 were categorized under the glycoside hydrolase (GH) family, which is crucial for the hydrolysis of glycosidic bonds in carbohydrates, this family includes the BGLs.

3.6.3 Screening for BGLs using BLAST

To identify potential BGL candidates within the GH family, the sequences were further analyzed using the BLAST, specifically targeting BGLs in eukaryotic organisms [18]. This analysis resulted in the identification of 27 sequences that showed significant similarity to known BGLs, suggesting their potential role in *Talaromyces-358's* lignocellulose degradation pathway.

3.6.4 Signal Peptide Prediction

Subsequent analysis using SignalP, a tool for predicting the presence of signal peptides, identified that 11 of the 27 BGL-like sequences contained signal peptides [19]. Signal peptides are indicative of proteins that are secreted or targeted to specific cellular compartments, which

is a desirable feature for enzymes involved in biomass processing as they are likely to be extracellular.

3.6.5 Determination of Molecular Weight

Bioinformatic tools were employed to estimate the protein molecular weights of the identified BGL sequences [20]. Among the sequences analyzed, one particular sequence was highlighted due to its molecular weight of 30.70 kDa and the presence of a signal peptide. This sequence matched the molecular weight of a potential BGL identified in native PAGE analysis, suggesting it could be a robust candidate for biorefinery applications.

Table 2: *List of potential BGLs.*

Gene ID	CAZy Family	SignalP	Mol. Wt.
g10460.t1	GH1	NO_SP	56.24 kDa
g325.t1	GH1	NO_SP	56.46 kDa
g7295.t1	GH1	NO_SP	57.07 kDa
g12652.t1	GH1	NO_SP	32.51 kDa
g11292.t1	GH132	SP	44.42 kDa
g10142.t1	**GH16**	**SP**	**30.70 kDa**
g7817.t1	GH16	NO_SP	34.35 kDa
g4370.t1	GH17	SP	56.66 kDa
g5686.t1	GH3	SP	84.47 kDa
g12783.t1	GH3	SP	84.00 kDa
g11624.t1	GH3	SP	91.88 kDa
g5588.t1	GH3	SP	93.20 kDa
g3987.t1	GH3	SP	93.32 kDa
g1562.t1	GH3	SP	90.64 kDa
g3540.t1	GH3	NO_SP	52.68 kDa
g3228.t1	GH3	NO_SP	94.00 kDa
g355.t1	GH3	NO_SP	91.59 kDa
g8213.t1	GH3	NO_SP	108.64 kDa
g4117.t1	GH3	NO_SP	92.16 kDa
g4590.t1	GH3	NO_SP	91.27 kDa
g8767.t1	GH3	NO_SP	92.53 kDa
g5763.t1	GH3	NO_SP	67.12 kDa
g9523.t1	GH3	NO_SP	63.01 kDa
g6050.t1	GH3	NO_SP	66.24 kDa
g8043.t1	GH31	SP	110.74 kDa
g9937.t1	GH31	SP	100.80 kDa
g7600.t1	GH31	NO_SP	103.51 kDa

3.7 Conclusion

The application of advanced protein analysis techniques, including SDS-PAGE and Native PAGE, has provided valuable insights into the expression and activity of beta-glucosidase (BGL) enzymes from *Talaromyces-358*. The results demonstrate the presence of distinct protein bands corresponding to active BGL enzymes in both glucose and xylan solutions. Genome analysis identified several potential BGL sequences, with one sequence showing strong potential for biotechnological applications due to its molecular weight and the presence of a signal peptide. These criteria match observations from the PAGE analysis suggesting that this sequence is likely to be effectively secreted under industrial conditions. These findings highlight the promising future biotechnological applications of *Talaromyces-358*, particularly in biomass conversion processes. The integration of protein expression profiles and genomic data paves the way for further optimization and development of efficient biorefinery relevant enzymes.

Summary and Future Perspectives

Summary

This study investigated the production and characteristics of acidophilic fungal beta-glucosidases (BGLs) from *Talaromyces* sp. ASS 358-9 (*Talaromyces-358*) for potential biorefinery applications. The research aimed to identify robust BGL enzymes suitable for bioconversion processes, particularly those involving lignocellulosic biomass, which often yields acidic fractions that can inhibit conventional enzymes.

The initial phase focused on the production of BGLs by culturing *Talaromyces-358* in various liquid media containing different substrates, including glucose, xylan, spruce liquor, and combinations thereof. Results indicated that glucose was the most favorable substrate for BGL production, showing consistent increases in enzyme activity over a 15-day cultivation period. The presence of spruce, while initially inhibitory, allowed *Talaromyces-358* to adapt and eventually produce significant BGL activity, demonstrating the organism's potential to overcome complex and inhibitory substrates. These findings suggest that *Talaromyces-358* is a promising candidate for biorefinery applications, given its ability to adapt to various substrates and conditions.

Physico-chemical characterization of the secreted BGLs revealed that enzyme activity peaked at 80°C, with considerable activity maintained at 90°C. Optimal pH conditions for BGL activity varied depending on the substrate used for the enzymatic assay, generally showing highest activity in slightly acidic environments (pH 4 to 6). This adaptability to high temperatures and acidic conditions is particularly advantageous for industrial processes that involve harsh operational settings.

Further, protein analysis using SDS-PAGE and Native PAGE identified distinct protein bands correlating with active BGL enzymes in glucose and xylan media. Genome analysis of *Talaromyces-358* highlighted several potential BGL sequences, with one sequence showing

strong potential due to its molecular weight and presence of a signal peptide, aligning with the protein bands observed in PAGE analysis.

Future Perspectives

Future research should focus on the following areas to fully realize the potential of *Talaromyces-358* BGL enzymes in industrial applications:

Optimization of Enzyme Production: Further studies should aim to enhance the product yields of BGL enzymes through genetic modifications such as overexpressing transcription factors that regulate cellulase gene expression or optimization of growth conditions.

Substrate Specificity: Investigating the enzyme's performance on a wider range of substrates, such as different types of lignocellulosic biomass including agricultural residues, hardwoods, and softwoods, can provide deeper insights into its versatility and potential for broader applications. By doing so, we can determine the enzyme's efficiency in breaking down complex carbohydrates under different conditions, which is crucial for optimizing its use in various industrial processes, such as biofuel production and other bioconversion applications.

Scale-Up Studies: Conducting pilot-scale studies to assess the practical feasibility of using *Talaromyces-358* BGL enzymes in industrial biorefineries. This includes evaluating the cost-effectiveness and efficiency of the enzyme production process.

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