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EXPRESSION AND CHARACTERIZATION OF A THERMOSTABLE α-GLUCURONIDASE FROM *Geobacillus kaustophilus*

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Abstract: Fossil fuels are a crucial resource for the global economy, but they also contribute to greenhouse gas emissions and environmental pollution. Lignocellulosic biomass, which includes cellulose, hemicellulose, and lignin obtained from plants, is a promising alternative to fossil fuels. It can help address these problems while reducing environmental impact. Enzymatic pre-treatment is used to degrade lignocellulosic biomass into subunits. The degradation of the hemicellulose structure involves accessory enzymes of industrial importance, such as α-glucuronidase. α-glucuronidases (EC 3.2.1.139) catalyze the hydrolysis of the α-1,2-glycosidic bond between α-D-glucuronic acid (GlcA) or its 4-o-methyl ether form (MeGlcA) and d-xylose units in the structure of xylooligosaccharides. The aim of this study was cloning, heterologous expression and biochemical characterization of the α-glucuronidase enzyme from the thermophilic bacterium *Geobacillus kaustophilus*. With this aim, the codon optimized α-glucuronidase gene was cloned into pQE-30 vector, overexpressed in *E. coli* BL21 (DE3), and purified with nickel affinity chromatography. The biochemical characterization of the purified α-glucuronidase revealed that the enzyme has activity at elevated temperatures between 65-90 °C. Additionally, *Geobacillus kaustophilus* α-glucuronidase enzyme showed higher activity at acidic pH values from pH 4.0 to 6.5. This is the first study to report the gene cloning, recombinant expression and biochemical characterization of α-glucuronidase which could be used as accessory enzyme from a thermophilic bacterium *Geobacillus kaustophilus*.

Keywords: *Geobacillus kaustophilus*, α-glucuronidase, Thermostable enzymes, Lignocellulosic biomass, Hemicellulolytic enzymes

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1. Introduction

Lignocellulosic biomass has gained popularity in recent years as a fuel for energy production due to the depletion of fossil fuels and climate change (Wang et al., 2021). Lignocellulosic biomass is the most abundant resource on our planet, with more than 40 million tons of non-food plant material produced each year. Together with forestry and agricultural wastes, this amounts to approximately 200 billion tons (Jaramillo et al., 2015). Numerous different agricultural goods are produced in significant amounts in the agricultural industry, and depending on the agricultural products, an equivalent quantity of agricultural waste is produced (Østby et al., 2020). The interest in this kind of waste is growing since it is employed in numerous sectors, including the culinary, chemical, and pharmaceutical industries. The development of novel bioproducts is made possible by the increased passion and interest in lignocellulosic biomass, a renewable biopolymer (Lee et al., 2014). Currently, the manufacture of bioethanol is the main application for lignocellulosic biomass, but it may also be used to make a variety of other products, including enzymes, food additives, pharmaceuticals, and cosmetic compounds (Jaramillo et al., 2015; Maitan-Alfenas et al., 2015; Arevalo-Gallegos et al., 2017).

The liberation of simple sugars in the lignocellulosic biomass structure is crucial for the production of high value-added products. However, this process is challenging due to the intricate nature of the biomass (Ezeilo et al., 2017). The hemicellulose chain of lignocellulose biomass consists of sugar molecules including xylose, galactose, and glucuronic acid which creates a significant obstacle for the direct hydrolysis of cellulose. Due to the structural diversity of xylans, the complex hydrolysis of xylan engages accessory enzymes such as, α-glucuronidases, α-L-arabinofuranosidases, acetyl xylan esterases, feruloyl esterases, and glucuronyl esterases, in addition to endoxylenases and β-xylosidases (Mohapatra and Manoj, 2019; Akkaya et al., 2023).

Hardwoods, conifers, and many cereals contain xylan bonded to glucuronic acid or methyl-glucuronic acid (Yan et al., 2017). Therefore, to completely use glucuronoxylans, glucuronidases (E.C. 3.2.1.131), which remove bound glucuronic acid and its derivatives from xylan, are required (Wang et al., 2016). Glucuronidases belong to the families of glucoside hydrolases GH4, GH67, and GH115 (Rogowski et al., 2014; Wang et al., 2016). While some of them act only on short xylooligomers or small model molecules, others hydrolyze glucuronic acid from polymeric xylan (Adıgüzel, 2013; Chong et al.,

2015).

α-glucuronidases (EC 3.2.1.139) catalyze the hydrolysis of the α-1,2-glycosidic bond between α-D-glucuronic acid (GlcA) or its 4-o-methyl ether form (MeGlcA) and d-xylose units in the structure of xylooligosaccharides. The majority of α-glucuronidases that catalyze the hydrolysis of this linkage belong to the glycoside hydrolase 67 (GH67) family (Septiningrum et al., 2015). Glycoside hydrolases are grouped according to their amino-acid sequence similarity (Aalbers et al., 2015). The presence of 4-O-methylglucuronic acid moieties is one of the main factors affecting the adsorption of the xylan chain, and treatment of xylan with α-glucuronidase significantly increases the level of adsorption of xylan (Chimphango et al., 2016).

Considering that many industrial and biotechnological processes take place under very harsh conditions, extremophilic microorganisms living under extreme conditions such as high temperature, high pressure, low/high pH, etc. are a great source of new enzymes that can be used in such processes (Demirjian et al., 2001; Van den Burg, 2003). The discovery of enzymes capable of operating under extreme conditions has enabled the development of new industrial processes (Demirjian et al., 2001). Enzymes obtained from thermophilic organisms have many advantageous in enzymatic hydrolysis of biomass (Yeoman et al., 2010). In general, thermostable enzymes have higher specific activity, so that the bioconversion process can be completed with a small amount of enzyme. They have high stability and can be used for a long time. In addition, better substrate solubility and enzyme penetration, as well as lower contamination, are achieved when conversion takes place at high temperatures (Turner et al., 2007; Yeoman et al., 2010).

In this study, we identified, cloned and expressed for the first time the α-glucuronidase enzyme encoded in the genome of the thermophilic bacterium *Geobacillus kaustophilus*. In addition, the biochemical characterization of the purified α-glucuronidase enzyme was performed.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich, Merck, and Biobasic unless otherwise stated. Salt-free oligonucleotides were purchased from Sentebiolab (Ankara, Türkiye). Enzymes were purchased from ThermoFisher Scientific unless otherwise stated.

2.2. Cloning of the *Geobacillus kaustophilus* α-Glucuronidase Gene

The codon optimized aguA gene of the *Geobacillus kaustophilus* (Uniprot ID: A0A0D8BWB2) was amplified by PCR (94 °C for 3 min, 1 cycle; 94 °C for 60 s/55-65 °C for 60 s/72 °C for 180 s, 30 cycles; 72 °C for 10 min, 1 cycle) using the forward primer *(5'-ATAGGTACCATGACGGCGGGATACGAACC-3')* and the reverse primer (*5'-CATAAGCTTTCACCGATAAATTTTCCGCCCG-3'*). The amplified product was digested with *Kpn*I and *Hind*III and then ligated with T4-DNA Ligase into pQE-30 plasmid vector. The ligation products were transformed into *E. coli* DH5α and positive transformants were validated with colony PCR and sequencing.

2.3. Heterologous Expression of the *Geobacillus kaustophilus* α-Glucuronidase Enzyme

The pQE-30 plasmid harboring α-glucuronidase gene was transformed into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) was inoculated into LB media supplemented with ampicillin (100 µg/mL) as pre-culture and cultivated overnight at 37 °C and 165 rpm. 0.5 mL of the pre-culture was inoculated into 50 mL of LB media supplemented with ampicillin (100 µg/mL) and cultivated at 37 °C, 165 rpm until OD600 reached 0.6-0.8. Protein expression was induced by the addition of 0.1 mM IPTG and 0.1 g/L Thiamine HCl. Then protein expression was carried out at 37 °C, 165 rpm for 24 h. Cells were harvested by centrifugation (4 °C, 3220 g, 30 min) and stored at -20 °C until further use.

2.4. Protein Purification and Quantification

The cell pellet was resuspended in four volumes of pH 7.5 phosphate buffer and lysed by sonication for 5 min (30 sec. on + 15 sec. off, 40% amplitude). The cell lysate containing α-glucuronidase enzyme was clarified by centrifugation (13680 g, 30 min, 4 °C). HisPurTM Ni-NTA Purification Kit was used to purify the His-tagged α-glucuronidase enzyme. The purified protein was dialyzed for 24 hours and then analyzed by SDS-PAGE and quantified using the Pierce™ BCA Protein Assay Kit.

2.5. Activity Measurement and Characterization of the *Geobacillus kaustophilus* α-Glucuronidase Enzyme

α-glucuronidase enzyme activity was measured via 4-nitrophenol based spectrophotometric assay. 56 µl of phosphate buffer (50 mM, pH 7.5), 40 µl of enzyme, and 4 µl of 50 mM p-nitrophenyl-α-D-glucuronide were added to the reaction medium and incubated at 37 °C for 20 min. After incubation, 100 µl of of 1 M Na_2CO_3 was added to the reaction medium to stop the reaction and absorbance was recorded at 400 nm. A previously prepared 4-nitrophenol standard graph (0.01-0.1 mM) was used for the determination of enzyme activity. One unit of enzyme activity was defined as the conversion of 1 µmol of substrate per minute. All measurements were performed in triplicate.

Optimal temperature, optimal pH and buffer were determined for further characterization of the *Geobacillus kaustophilus* α-glucuronidase enzyme. Additionally, effects of various chemicals were tested on enzyme activity. First, the optimal temperature was determined using the activity test explained above at various temperature between 40 and 90 °C. Then optimal pH and assay buffer was determined by testing 50 mM sodium acetate buffer (pH 4.0-6.0), 50 mM phosphate buffer (pH 6.0-7.5), 50 mM tris buffer (pH 7.5-9.0), and 50 mM glycine buffer (pH 9.0-10.0). Lastly, 10 mM of mM

SDS, EDTA, $FeCl_3$, $MgCl_2$, KCl, $CaCl_2$, $MnCl_2$, ethanol, DMSO, 2-Mercaptoethanol, DTT, sodium citrate, and $CoCl_2$ was included in the reaction mixture to determine the effect of various chemicals on α-glucuronidase enzyme activity.

3. Results and Discussion

3.1. Cloning of the *Geobacillus kaustophilus* α-Glucuronidase Gene

Geobacillus kaustaphilus is a thermophilic, gram-positive bacterium that was first isolated from the Mariana Trench. It grows optimally between 55 °C and 65 °C and can tolerate temperatures between 37 °C and 75 °C. The bacterium can also grow in a pH range of 6 to 8, with optimal growth occurring between pH 6.2 and 7.5.

To express the *Geobacillus kaustaphilus* α-glucuronidase enzyme heterologously in *E. coli*, the relevant gene codon was first optimized and synthesized as a synthetic gene by Genscript company. Subsequently, PCR was used to obtain copies of the gene and add restriction recognition sites for ligation to the pQE-30 expression vector. To determine the optimal annealing temperatures of the primers, gradient PCR was performed between 55 and 65 °C. The resulting PCR product was then analyzed on a 1% agarose gel (Figure 1). The α-glucuronidase gene yielded a 2040 bp. product through the PCR process at all tested temperatures, as shown in the figure.

The 2040 bp. gene fragment was digested with *KpnI* and *HindIII* and ligated into pQE-30 vector digested with the same restriction enzymes. The obtained plasmid construct was transformed into *E.coli* DH5α. Positive transformants were validated by colony PCR (Figure 2) and sequencing. The pQE-40 vector harboring the α-glucuronidase gene was transformed into *E. coli* BL21 (DE3) for heterologous expression.

3.2. Heterologous Expression and Purification of Recombinant α-glucuronidase Protein

Geobacillus kaustaphilus α-glucuronidase enzyme was expressed in *E. coli* BL21 (DE3) and purified with His-Tag purification. Purified protein fractions were analyzed by SDS-PAGE (Figure 3) with a molecular mass of approximately 78.5 kDa. Purified protein fractions were combined and dialyzed to remove imidazole from enzyme solution. The concentration of the purified α-glucuronidase enzyme was determined using the Pierce BCA Protein Assay Kit after dialysis. A standard graph, containing bovine serum albumin (BSA) ranging from 0-2000 µg/mL, was used for protein quantification. The BCA analysis revealed that the concentration of the purified α-glucuronidase enzyme was 76.04 µg/mL.

3.3 Characterization of Recombinant *Geobacillus kaustaphilus* α-glucuronidase Enzyme

The recombinant *Geobacillus kaustaphilus* α-glucuronidase enzyme was characterized by determining the temperature, buffer, and pH value at which it showed optimum activity. Additionally, the effects of certain chemicals on enzyme activity were examined. The α-glucuronidase enzyme was characterized using a 4-nitrophenol (pNP) based colorimetric activity assay. The colorimetric activity determination utilized the pNP standard graph prepared at concentrations ranging from 0.001 mM to 0.1 mM. To characterize the α-glucuronidase enzyme, we incubated the enzymatic reaction at 40 °C for 20, 30, and 40 minutes to determine the optimal reaction time. We also tested two different buffers, phosphate and HEPES, for the reaction. Spectroscopic measurements were taken at 405 nm to determine the amount of pNP released, as shown in Figure 4. Based on the results, it was determined that the optimal conditions were achieved with a 30-minute incubation in phosphate buffer.

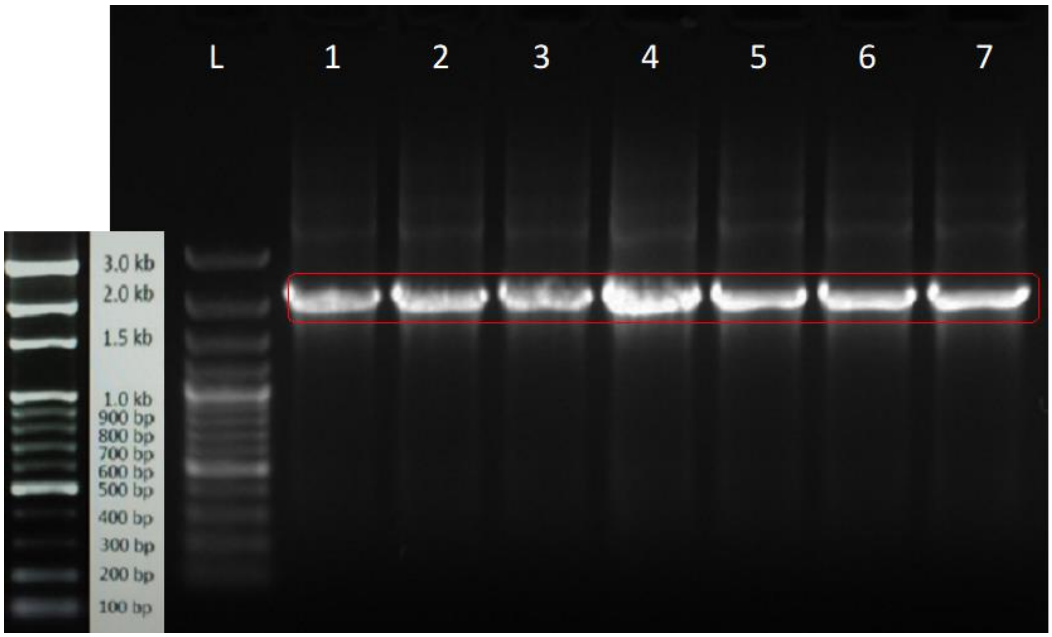

Figure 1. Agarose gel analysis of PCR performed for the amplification of α-glucuronidase gene. L: Molecular weight marker, 1: 55 °C, 2: 56.5 °C, 3: 57.6 °C, 4: 59 °C, 5: 60.7 °C, 6: 63.3 °C, 7: 64.7 °C.

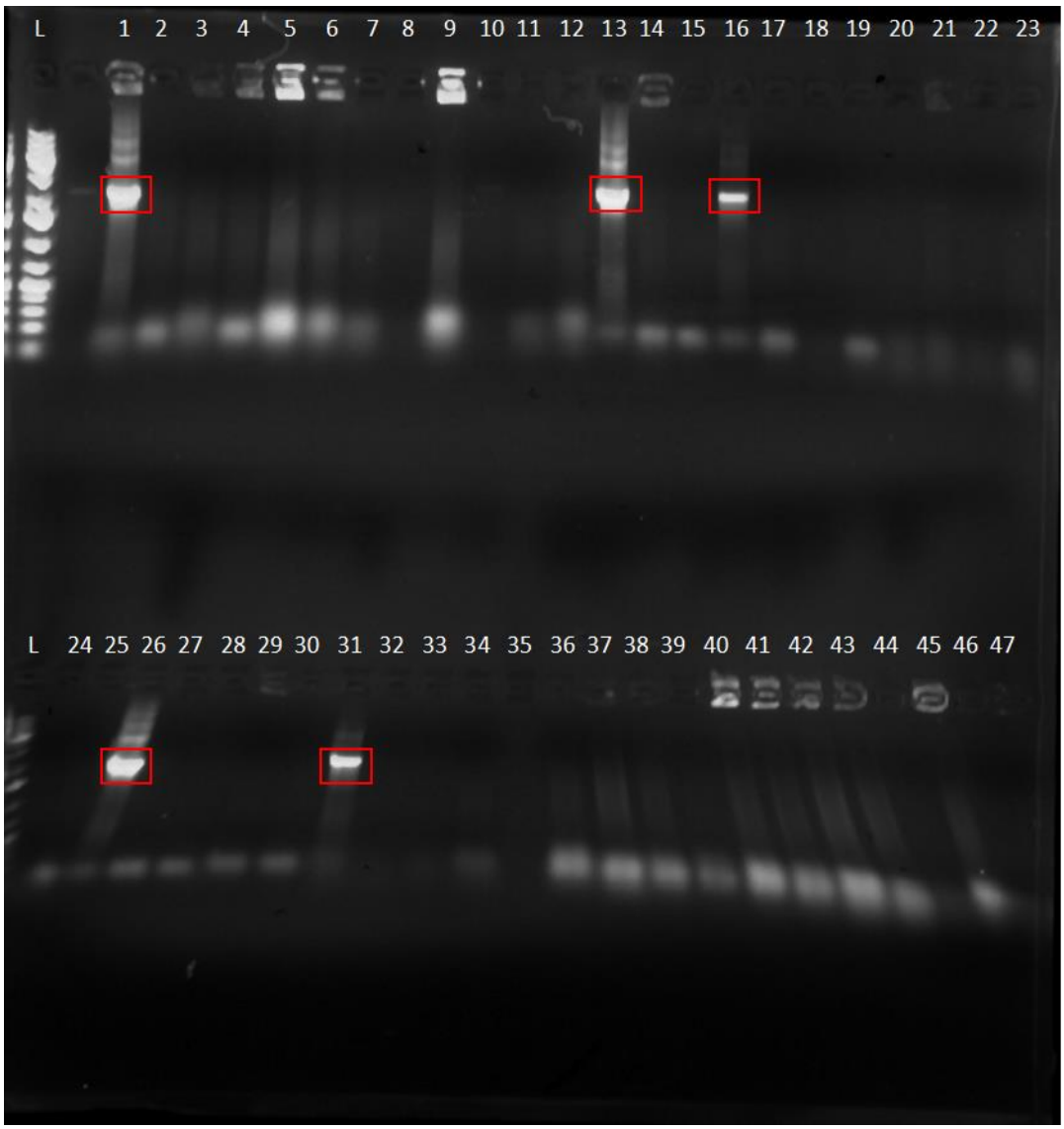

Figure 2. Agarose gel image of colony PCR analysis for α-glucuronidase gene. L: Molecular weight marker, 1-47: different colonies obtained after transformation. Red boxes show the positive clones with 2 kb DNA band.

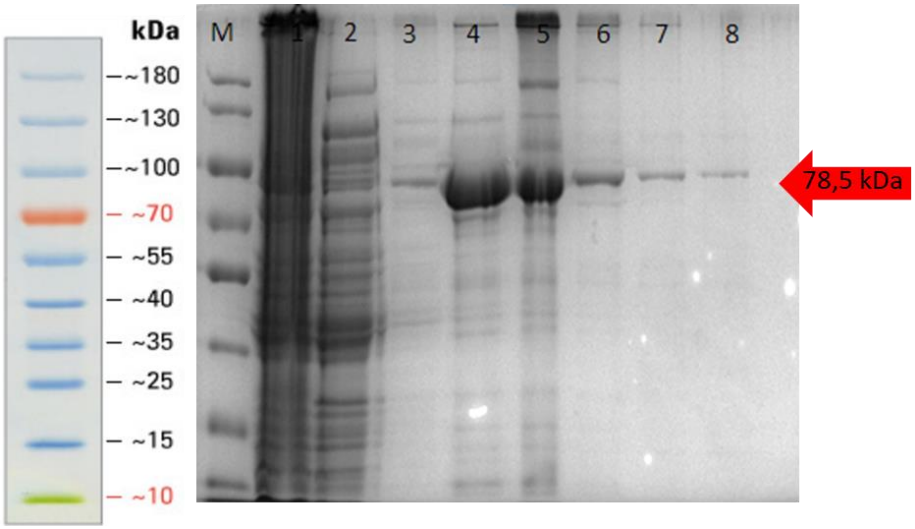

Figure 3. SDS-PAGE analysis of α-glucuronidase enzyme. M: Marker, 1: Cleared supernatant, 2: Flow-through (FT), 3: Wash Fraction, 4: Elution Fraction 1 (100 mM imidazole), 5: Elution Fraction 2 (200 mM imidazole), 6: Elution Fraction 3 (300 mM imidazole), 7: Elution Fraction 4 (400 mM imidazole), 8: Elution Fraction 5 (500 mM imidazole).

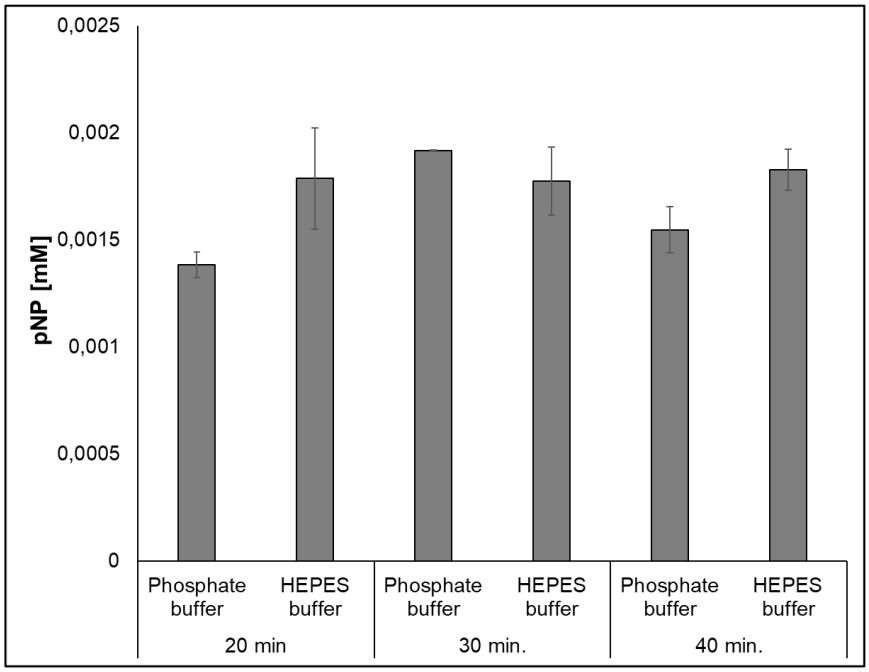

Figure 4. Determination of the appropriate incubation time for α-glucuronidase enzyme activity assay.

The results obtained after 30 minutes of incubation in phosphate buffer were the most consistent compared to other time periods and HEPES buffer. Therefore, 30 minutes of incubation in phosphate buffer was selected as the optimum reaction time, and subsequent biochemical characterization procedures were carried out. The standard deviation of the other results was higher than that obtained with 30 minutes of incubation in phosphate buffer.

The biochemical characterization of the recombinant α-glucuronidase enzyme began by determining its optimum temperature. Enzymatic activity was measured at various temperatures ranging from 40 °C to 90 °C, and the amount of pNP released at 405 nm was then measured spectrophotometrically. The temperature at which the highest enzymatic activity was measured is considered 100%, and the activity values obtained at other temperatures were proportioned accordingly (see Figure 5). The optimum activity of the α-glucuronidase enzyme was observed at 75 °C. Enzyme activity increased gradually from 45 ° and decreased slightly after 75 °C. The enzyme exhibited 66% activity at 70 °C and 40% activity at 65 °C when the activity observed at 75 ºC was evaluated as 100%. Additionally, the enzyme activity remained around 10% at moderate temperatures (between 40-50 °C). At higher temperatures, the enzyme retained 93% of its activity at 80 °C and 90% at 85 °C, while exhibiting about 80% activity at 90 °C. The study's findings indicate that the enzyme is thermophilic, as expected, and exhibits low activity at mesophilic temperatures. Suresh and coworkers reported that the α-glucuronidase enzyme derived from *Thermotoga maritima*, a hyperthermophilic bacterial species, demonstrated the highest activity at 60 °C (Suresh et al., 2003). Similarly, Shao et al. (1995) found that the optimal temperature for the enzyme was 60 °C in their study with α-glucuronidase enzyme obtained from *Thermoanaerobacterium sp*.. Zaide and coworkers determined that the optimum temperature for *Bacillus stearothermophilus* α-glucuronidase enzyme activity was 75 °C (Zaide et al., 2001). In a similar study, the optimum temperature for *Geobacillus stearothermophilus* α-glucuronidase gene activity was found to be 65 °C (Dalia et al., 2004). In this study, we observed that the recombinantly produced *Geobacillus kaustaphilus* α-glucuronidase enzyme shares similar characteristics with those obtained from other thermophilic Bacillus species. Furthermore, it was found to have a higher operating temperature than Thermotoga and Thermoanaerobacter species, which are also thermophilic organisms described in the literature. Additionally, there are α-glucuronidase enzymes identified from mesophilic bacteria in the literature. The optimal activity of the α-glucuronidase enzyme obtained from a mixed culture was found to be within the range of 45 °C (Lee et al., 2012). Furthermore, the optimal operating temperature of the α-glucuronidase enzyme obtained from *Aspergillus fumigatus* was determined to be 37 °C (Rosa et al., 2013).

After determining the optimal temperature for α-glucuronidase enzyme, we conducted enzyme activity tests using different buffer systems and pH values. The enzyme was tested using sodium acetate at pH 4 to 6, phosphate buffer at pH 6 to 7.5, tris buffer at pH 7.5 to 9, and glycine buffer at pH 9 to 10 to determine the pH value and buffer system that resulted in the highest enzyme activity. The pH value with the highest enzymatic activity was considered 100%, and the activity values obtained at other pH values were adjusted proportionally (see Figure 6).

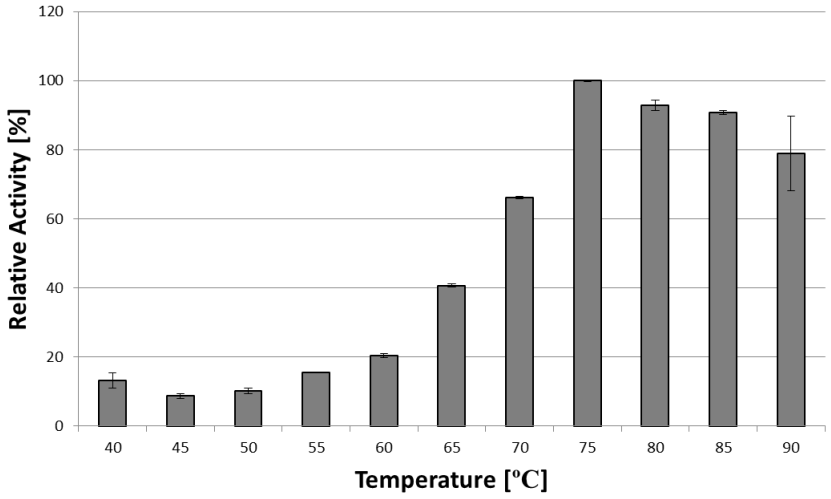

Figure 5. Effect of temperature on α-glucuronidase enzyme activity. The activity assay was performed in pH 7.5 phosphate buffer at various temperatures for 30 min.

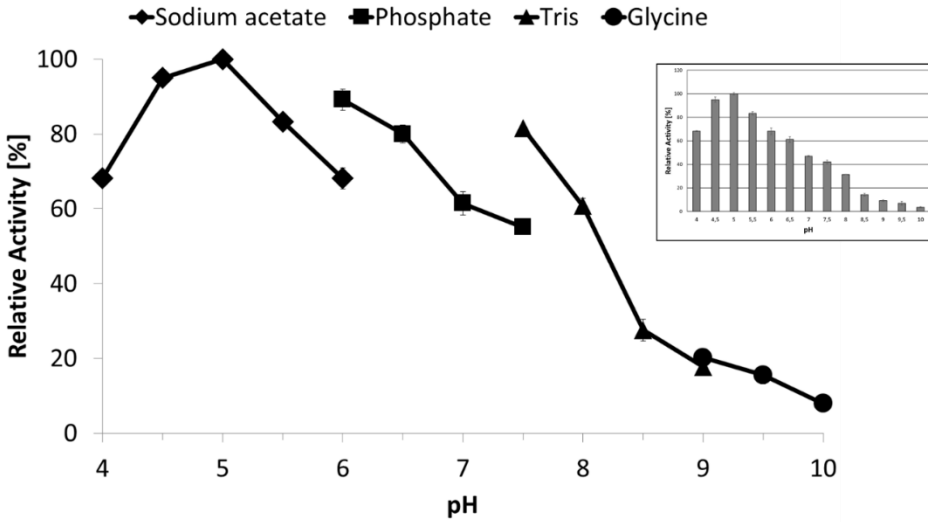

Figure 6. Effect of buffer and pH on α-glucuronidase enzyme activity. The activity was measured at 75 °C in various buffers of pH 4.0 – 10.0 for 30 min.

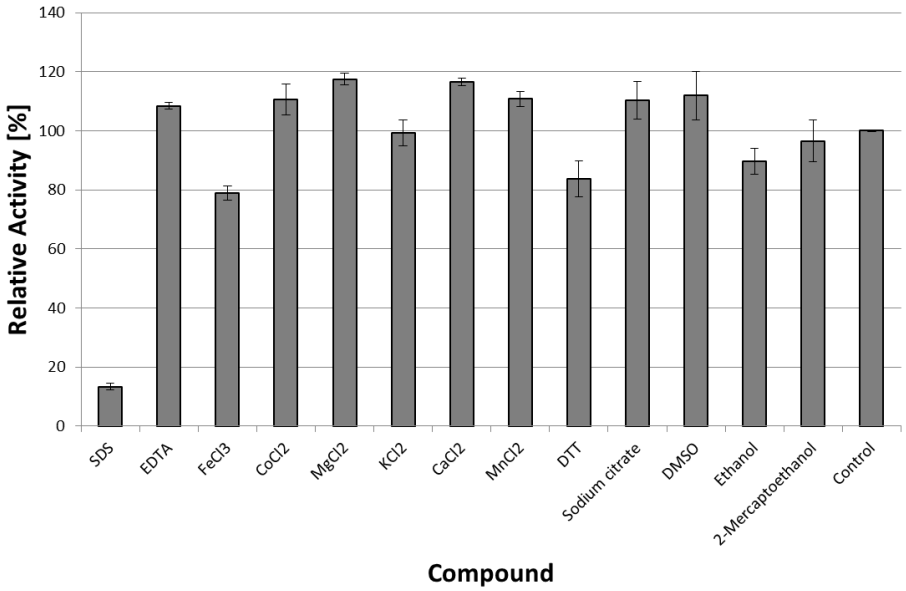

Figure 7. Effect of chemicals on α-glucuronidase enzyme activity. Chemical compounds were added to the reaction medium at final concentration of 0.1 mM.

The results indicate that the optimal pH value for the α-glucuronidase enzyme is pH 5.0, and it exhibits the highest activity in a sodium acetate buffer. The enzyme activity increased between pH 4.0 and 5.0 but started to decrease after pH 5.0. The α-glucuronidase enzyme from *Geobacillus kaustaphilus* showed the highest activity (over 80%) in mildly acidic conditions and about 50% activity at neutral pH values. In basic conditions, the α-glucuronidase enzyme lost most of its activity and exhibited about 10% activity. These findings indicate that the α-glucuronidase enzyme from *Geobacillus kaustaphilus* is both acidophilic and thermophilic. The optimal pH values for α-glucuronidase enzymes from *Aspergillus fumitatus*, *Thermotoga maritima*, and *Thermoanaerobacterium sp*. were determined to be 5.0, 7.5, and 7.5, respectively (Shao et al., 1995; Suresh et al., 2003; Rosa et al., 2013). According to Zaide et al. (2001) the optimal activity of the thermophilic *Bacillus stearothermophilus* α-glucuronidase enzyme was achieved in a phosphate buffer with a pH of 7.0. When comparing the results of this thesis study to other α-glucuronidase enzymes described in the literature, it was observed that enzymes described as thermophilic in the literature exhibited activity at neutral pH values. The α-glucuronidase enzyme produced in this study by recombinant *Geobacillus kaustaphilus* is both acidothermophilic and distinct from other thermophilic relatives in the literature. This acidophilic and thermophilic feature makes the *Geobacillus kaustaphilus* α-glucuronidase enzyme superior to its competitors, especially in industrial processes that occur under extreme conditions.

After determining the optimal operating temperature, pH value, and buffer for the α-glucuronidase enzyme, the aim was to evaluate the impact of different chemicals on enzyme activity. Under optimum conditions, the enzymatic reaction was carried out with the addition of SDS, EDTA, $FeCl_3$, $CoCl_2$, $MgCl_2$, KCl_2, $CaCl_2$, $MnCl_2$, DTT, sodium citrate, DMSO, ethanol, and 2-mercaptoethanol at a concentration of 0.1 mM. The amount of pNP released at 405 nm was measured spectrophotometrically. After the reaction, enzyme activity was measured and compared to a control reaction without any chemicals. The results, shown in Figure 7, indicate that $MgCl_2$ and $CaCl_2$ increased enzyme activity by approximately 20%. Conversely, $FeCl_3$, DTT, and ethanol partially inhibited enzyme activity, while SDS inhibited it by about 85%. No significant effect on enzyme activity was observed for any other chemicals. The addition of EDTA, $CoCl_2$, $MgCl_2$, $CaCl_2$, $MnCl_2$, sodium citrate, and DMSO positively affected α-glucuronidase activity. The highest increase in activity, approximately 117%, was observed with $MgCl_2$ and $CaCl_2$, while the increase in activity for the other chemicals remained around 110%. KCl_2 and 2-mercaptoethanol did not show any effect. The addition of ethanol resulted in an activity of 90%, while the addition of DTT and $FeCl_3$ resulted in activities of 83% and 79%, respectively. Out of the 13 chemical compounds tested, only SDS was found to be inhibitory, causing a dramatic decrease in enzyme activity to only 13% in a reaction medium containing 0.1 M SDS. The other chemicals did not cause any significant change. Zaide and coworkers also investigated the effect of $FeCl_3$, $CoCl_2$, $MgCl_2$, KCl, $CaCl_2$, $BaCl_2$, $NiCl_2$, $HgCl_2$, $ZnCl_2$, $AgNO_3$, and $CuSO_4$ on the activity of the *Bacillus stearothermophilus* α-glucuronidase enzyme. The enzyme activity was highly inhibited by $HgCl_2$, $ZnCl_2$, $AgNO_3$, and $CuSO_4$, while only $FeCl_3$ moderately increased it. The effect of other chemicals was not significant (Zaide et al., 2001).

4. Conclusion

The complete enzymatic hydrolysis of lignocellulose requires many cellulolytic and hemicellulolytic enzymes. While endoxylenase and β-xylosidase are primarily responsible for breaking down the hemicellulose structure, α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, acetylxylan esterase, and β-mannanase also play a role in its complete degradation. Glucuronic acid or its methyl ester is present in the lignocellulosic structure attached to xylan. During the hydrolysis of xylans, α-glucuronidases work alongside many other enzymes. α-glucuronidase enzymes are utilized in various industrial and biotechnological processes. Furthermore, this enzyme has applications in disease diagnosis and the food industry. In this study, the gene encoding α-glucuronidase enzyme in *Geobacillus kaustaphilus* was cloned into pQE-30 plasmid and successfully expressed in *E. coli* BL21 (DE3). The expressed enzyme was purified via nickel affinity chromatography and characterized biochemically. The purified enzyme showed the optimum activity at 75 °C and pH 5.0 in sodium acetate buffer. Furthermore, α-glucuronidase enzyme was inhibited in the presence of SDS, $FeCl_3$, DTT, and ethanol. On the other hand, EDTA, $CoCl_2$, $MgCl_2$, $CaCl_2$, $MnCl_2$, sodium citrate, and DMSO positively affected α-glucuronidase activity. In conclusion, the activity at high temperatures and mild acidic conditions shows the potential of the *Geobacillus kaustaphilus* α-glucuronidase enzyme in the lignocellulosic biomass degradation.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	H.T.	Y.A.
C	20	80
D	50	50
S		100
DCP	50	50
DAI	50	50
L	50	50
W	50	50
CR	20	80
SR	50	50
PM	50	50
FA		100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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