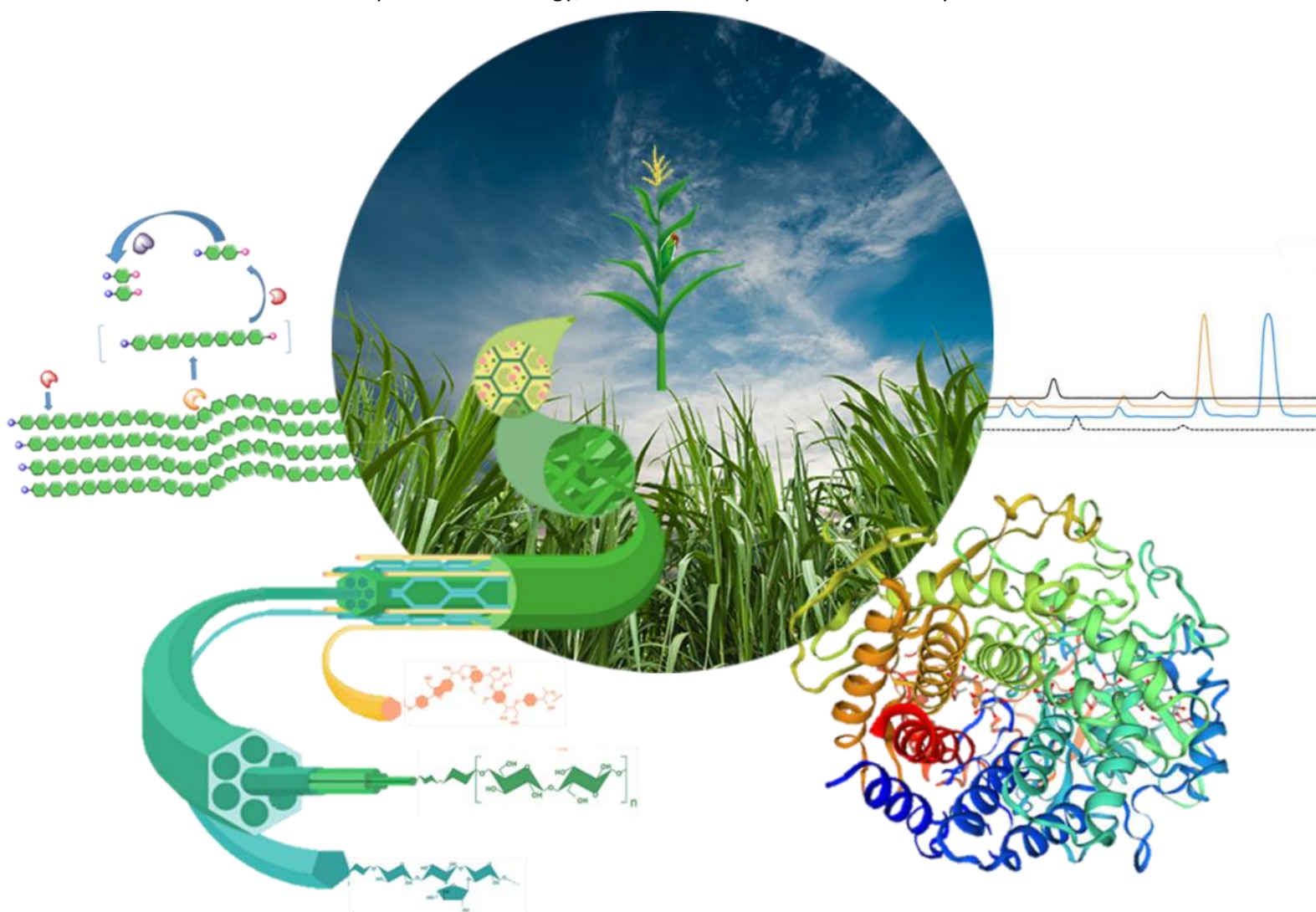




Study of the synergistic action of cellulolytic enzymes of a genetic locus of the bacterium *Bacillus safensis*

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Table of Acronyms

Microcrystalline Cellulose	MC
Carbohydrate-Binding Modules	CBMs
Carbohydrate Active Enzymes Database	CAZy
Lytic Polysaccharide Monooxygenases	LPMO
Auxiliary Activity	AA
1,4- β -D-glucan-cellobiohydrolases	CBH
endo-,4- β -D-glucanases	EGO
β -glucosidase	BG
Glycoside Hydrolases	GH
National Center for Biotechnology Information	NCBI
Menaquinone 7	MK-7
Spacecraft-Assembly Facility	SAF
Luria Bertani	LB
Polymerase Chain Reaction	PCR
Ultraviolet	UV
Molecular Weight cut-off	MWCO
Carboxymethyl Cellulose	CMC
Ethylenediaminetetraacetic Acid or Edetic Acid	EDTA
Sodium dodecyl-sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Phosphoric Acid Swollen Cellulose	PASC
High-performance anion-exchange Chromatography with pulsed amperometric detection	HPAEC-PAD
Isopropyl β - d-1-thiogalactopyranoside	IPTG
Bacterial cellulose	BC

Abstract

In this study, a β -1,4-exoglucanase of the GH48 protein family was characterized and its synergistic action with an endo-1,4- β -D-glucanase of the GH9 protein family and a β -glucanase of the GH5 protein family was examined. These three enzymes originate from a *Bacillus safensis* (ATHUBA63), which was isolated from a local soil sample. The *bsgh48* gene was cloned and overexpressed in competent cells of *Escherichia coli* BL21. From these bacterial cells, BsGH48 was isolated. The molecular weight of BsGH48 was estimated at 78.6 kDa. A bioinformatics analysis took place where the *bsgh48* sequence was compared with others from online databases and the protein's 3D-structure was predicted. The optimum conditions of action for BsGH48 were determined at 50 °C and pH 5.0. The effect of several modulators on the activity of BsGH48 was examined. The Mn^{2+} and Ca^{2+} divalent ions enhance substantially its activity. However, it is significantly reduced by both ferrous ions, Fe^{2+} and Fe^{3+} . BsGH48 was not affected by SDS or EDTA. The stability of the protein molecule was tested. From the results of the thermal stability assay the thermal inactivation energy ($E_{(a)d}$) of BsGH48 was calculated at 159.7 ± 7.3 kJ/mole. Its thermodynamic parameters of thermal deactivation were also evaluated. At the pH stability assay, BsGH48 showed great stability in the pH range of 5.0-9.0. The substrates that BsGH48 hydrolyzed are Avicel and PASC. The kinetic constants, K_m and V_{max} , indicated that BsGH48 presents a preference towards PASC as a substrate. Moreover, BsGH48 proved to be a non-processive enzyme. The hydrolysis products of PASC from BsGH48, BsGH9, and BsGH5 were analyzed with HPAEC-PAD, to set a backbone for the synergistic study. BsGH48's only product was cellobiose. BsGH9's products were cellobiose and glucose. BsGH5's products were cellobiose, cellotriose, and cellotetraose. BsGH9 presented the highest enzymatic activity. In the synergistic study, the enzymes were first paired up and then they were all included in the reaction mix. From their synergistic action, there were not produced any new products. Nevertheless, the enzymatic activity of all the enzymes and the hydrolysis of PASC were enhanced.

Keywords: cellulases, *Bacillus safensis*, β -1,4-exoglucanase, endo-1,4- β -D-glucanase, β -glucanase, GH48, GH9, GH5, synergism

Περίληψη

Στην παρούσα διπλωματική εργασία χαρακτηρίστηκε μια β-1,4-εξαγλουκανάση της οικογένειας 48 των γλυκοζυλ-υδρολασών και μελετήθηκε η συνεργιστική της δράση με μία ενδογλουκανάση της οικογένειας 9 και μία β-γλουκανάση της οικογένειας 5. Τα τρία αυτά ένζυμα ταυτοποιήθηκαν από το βακτήριο *Bacillus safensis* (ATHUBA63), το οποίο απομονώθηκε από δείγμα τοπικού χώματος. Το γονίδιο *bsgh48* κλωνοποιήθηκε και υπερεκφράστηκε σε δεκτικά κύτταρα *Escherichia coli* BL21, από όπου και απομονώθηκε το ένζυμο BsGH48. Το μοριακό βάρος του ενζύμου προσδιορίστηκε στα 78.6 kDa. Διεξάχθηκε βιοπληροφορική ανάλυση όπου η αλληλουχία του γονιδίου *bsgh48* συγκρίθηκε με άλλες πρωτεϊνικές αλληλουχίες από διαδικτυακές βάσεις δεδομένων και προσδιορίστηκε η τρισδιάστατη δομή του ενζύμου. Το BsGH48 παρουσιάζει θερμοκρασιακό βέλτιστο στους 50 °C και σε pH 5.0. Εξετάστηκε η επίδραση διαφόρων παραγόντων στην ενζυμική ενεργότητα του. Τα δισθενή ιόντα Mn^{2+} και Ca^{2+} φαίνεται να ενισχύουν σημαντικά την δράση του ενζύμου, σε αντίθεση με τα ιόντα σιδήρου, Fe^{2+} και Fe^{3+} , όπου και τα δύο την εξασθενούν. Το ένζυμο BsGH48 δεν επηρεάζεται από την παρουσία SDS ή EDTA. Μελετήθηκε η σταθερότητα του ενζύμου αυτού. Από τα αποτελέσματα της μελέτης της θερμικής σταθερότητας του BsGH48 υπολογίστηκε η ενέργεια θερμικής απενεργοποίησης ($E_{(a)d}$) στα 159.7 ± 7.3 kJ/mole. Ακόμα, υπολογίστηκαν και οι θερμοδυναμικές παράμετροι της θερμικής απενεργοποίησής του. Το ένζυμο BsGH48 παρουσίασε σημαντική σταθερότητα σε εύρος pH 5.0-9.0. Τα υποστρώματα τα οποία υδρολύθηκαν από το ένζυμο BsGH48 ήταν το Avicel και το PASC. Το ένζυμο αυτό παρουσιάζει προτίμηση στο υπόστρωμα PASC σύμφωνα με τις κινητικές σταθερές του, K_m και v_{max} . Επίσης, το BsGH48 αποδείχτηκε να είναι ένα «non-processive» ένζυμο. Τα προϊόντα υδρόλυσης του PASC από τα ένζυμα BsGH48, BsGH9 και BsGH5 αναλύθηκαν με τη χρήση HPAEC-PAD, προκειμένου να θέσουν τη βάση για την μελέτη της συνεργιστικής τους δράσης. Το BsGH48 είχε ως μοναδικό του προϊόν την κελλοβιόζη. Το BsGH9 είχε ως προϊόντα την κελλοβιόζη και τη γλυκόζη, ενώ το BsGH5 την κελλοβιόζη, την κελλοτριόζη και την κελλοτετραόζη. Η μεγαλύτερη ενζυμική ενεργότητα σημειώθηκε από το BsGH9. Κατά την συνεργιστική μελέτη τα ένζυμα πρώτα εξετάστηκαν ανά δύο και έπειτα συμπεριλήφθηκαν όλα μαζί στην ίδια πρωτεϊνική αντίδραση. Κατά τη συνεργιστική τους δράση δεν προέκυψε κάποιο καινούργιο προϊόν. Ωστόσο, ενισχύθηκε τόσο η ενζυμική δάση όλων των πρωτεϊνικών μορίων όσο και η υδρόλυση του υποστρώματος PASC.

INTRODUCTION

The rising concerns around global warming as well as the rapid depletion of fossil fuels constitute the development of diverse strategies aiming toward carbon neutrality a high priority issue. Carbon dioxide is utilized by biomass through photosynthesis. Thus, biomass-derived biorefineries can be used for the production of fuels, materials, and chemicals replacing petroleum-based refineries (Park et al. 2022). Circular carbon economies aim for a transition towards a more environmentally friendly system that supports and enables sustainable development and conserves economic growth. These economies are based on biomass as their most important feedstock.

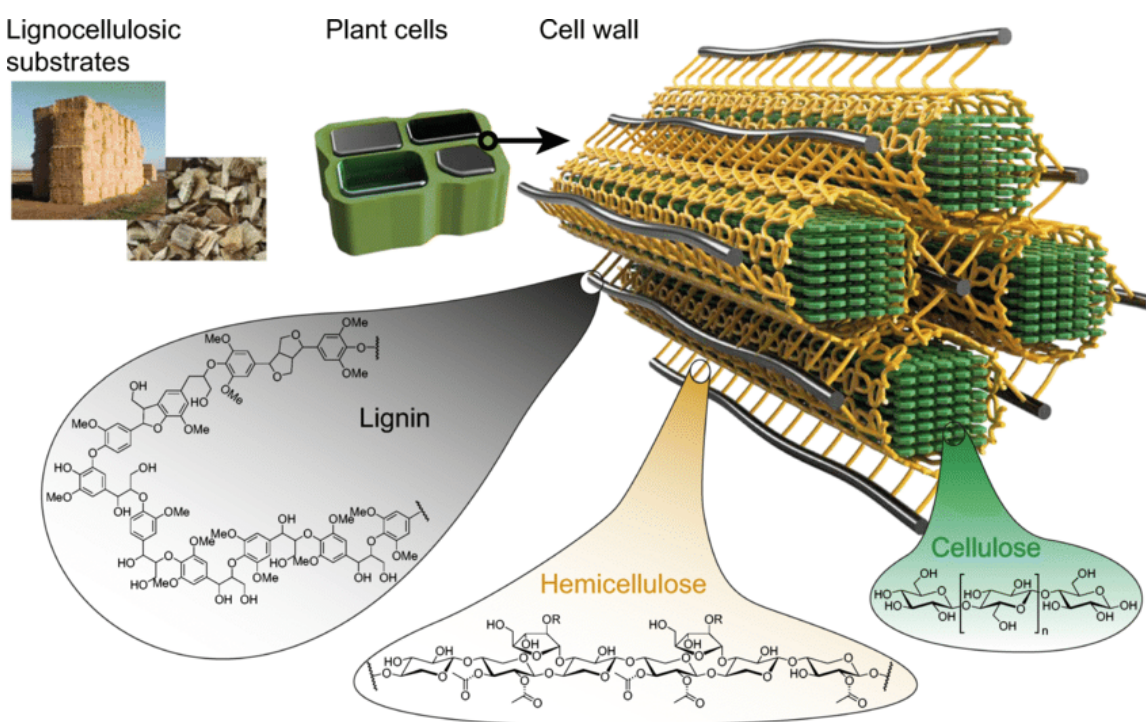


Figure 1. Lignocellulosic plant cell walls' structure and composition (Brethauer, Shahab, and Studer 2020).

Lignocellulose is the fibrous material in plant cell walls and the primary component of biomass. It comprises about half of the plant's dry matter. Lignocellulose constitutes the most abundant renewable resource on the planet. Especially the agricultural lignocellulosic wastes, because of their low prices, high availability, and wide distribution (Y.-J. Liu et al. 2020). There are numerous applications (bioprocesses and products) for the biotechnological conversion of this material. The use of lignocellulosic wastes remains a worldwide research hotspot and is attracting the attention of governments all around the world. It is necessary to replace about

25% (v/v) of transportation fuels with biofuels for energy security and reduction of greenhouse gas emissions. Consequently, the US Energy Independence and Security Act requires 36 billion gallons of biofuels to be produced by 2022. Concurrently, the European Union set a target of using up to 18% of the total agricultural land in the EU for biofuel production by 2030 (Council 2006; Sissine 2007). Lignocellulose's structural properties are dense, tough, and complex and thus it is a difficult substrate to degrade and utilize. It consists of 30–50% (w/w) cellulose, 10–40% hemicellulose, and 5–30% lignin. The degradation of lignocellulose can be achieved by synergistic mixtures of enzymes including cellulases, hemicellulose, and lignin-degrading. In nature, these enzymes are commonly secreted by microorganisms (You et al. 2023). Cellulose is the most abundant component in lignocellulose biomass. Cellulases are synthesized by a wide variety of microorganisms including both fungi and bacteria. The cellulolytic microorganisms can be aerobic, anaerobic, mesophilic, or thermophilic (Kuhad, Gupta, and Singh 2011).

1. Cellulose

The unique chemical structure of the fibrous component and the main molecule of all higher plant cells was named “cellulose” by Anselme Payen, in 1838. Anselme Payen was a French chemist who presented that a great variety of plant materials yield a fibrous substance after purification with acid-ammonia treatment and extraction with water, alcohol, and ether. However, the term ‘cellulose’ was coined by the French Academy, which evaluated his work. Cellulose can be produced by plants, algae, bacteria, fungi, protozoans, and animal tunicates. It is the most abundant substance in the biosphere, but it is never found alone, it is always associated with many other plant substances. Cellulose is a polymer of glucose units (D-glucose) with cellobiose indicated as its repeating unit. Cellobiose is 4-O- β -D-glucopyranosyl-D-glucose, which is a product of cellulose's hydrolysis. The crystallinity of this component is a result of the chemical compositions and conformation of cellulose chains combined with their hydrogen bonding system, and it appears comparable to cellulose from different sources.

Cellulose consists of D-glucopyranose ring units in the $4C_1$ -chair configuration. This configuration presents the lowest energy conformation and is independent of the source. These units are linked by β -1,4-glycosidic bonds and as a result, the macromolecule's axis is alternately turning by 180° . The hydroxyl groups of the D-glucose are found at C6, C3, and C2. Cellulose is formed by “polycondensation”, so the chain ends are chemically different. There are two types of chain end variations, the nonreducing end and the reducing end. The first one consists of an

anomeric C atom linked by glycosidic bonds and the second one contains a D-glucopyranose unit in equilibrium with the aldehyde function. The molecular structure of the cellulose chain may change if hydrolysis or oxidation occurs. These changes take place mainly on the surface of this fibrous component.

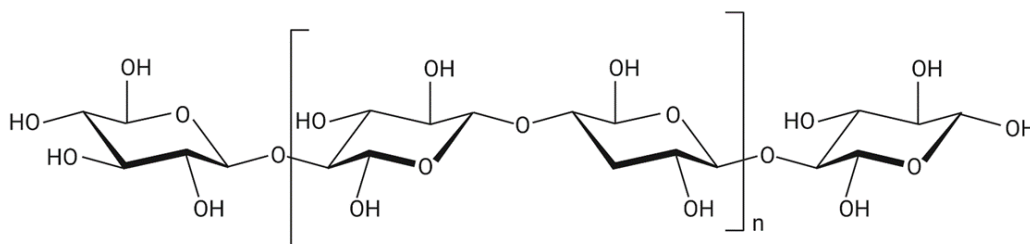


Figure 2. Cellulose chemical structure.

The hydrogen bonding system of cellulose has a great influence on its properties. More specifically, it presents a limited solubility in numeric solvents, a significant crystallinity, and its hydroxyl groups are reactive. The intramolecular and intermolecular hydrogen bonds form by the three hydroxyl groups, the ring's oxygen atoms of the D-glucopyranose unit, and the glycosidic linkage interactions within the chain or another one. The three-dimensional arrangements can be various. The hydrogen bonds play a part in the stiffness of cellulose, and when they are intramolecular, they are responsible for the strong interactions between its chains. As a result of these interactions, cellulose forms crystalline structures. Other than the crystalline domains, it possesses amorphous or noncrystalline domains, which affect its physical and chemical properties. The interactions that occur between cellulose, and enzymes, water, reactive or absorptive substances take place firstly at the noncrystalline domains.

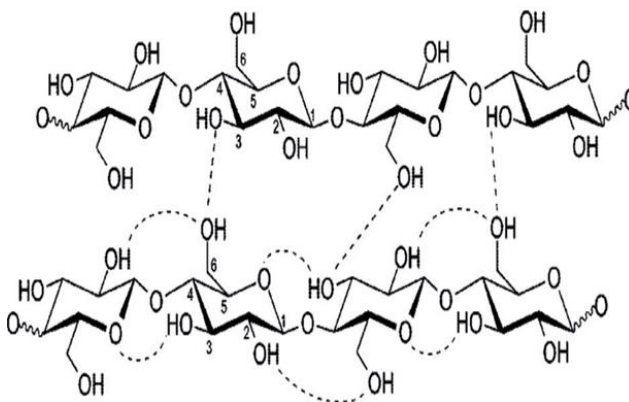


Figure 3. Hydrogen bond structure of cellulose (Dey and Dey 2021).

Cellulose as a substrate in Enzyme Biotechnology mainly appears in the form of microcrystalline cellulose (MC), such as Avicel. If solid lignocellulosic biomass is treated with aqueous hydroxide to remove other constituents and then acidic hydrolysis follows, MC is formed as a white and odorless crystalline powder. The less crystallized regions are the ones hydrolyzed. Thus, resulting in a solution of water-insoluble and crystalline residues (Marchessault and Sundararajan 1983; Heinze 2016; Wertz, Bédoué, and Mercier 2010).

2. Degradation of cellulose/ Cellulose hydrolysis

Generally, it was believed that the complete degradation of cellulose is accomplished by the enzymatic complex that includes endo- β -1,4-glucanases, cellobiohydrolases, and β -glucosidases. Endo- β -1,4-glucanases hydrolyze the cellulose chain internally. Cellobiohydrolases or exo-glycanases hydrolyze the ends of cello-oligosaccharides. β -glucosidases act on cellobiose, which are the monomers of the cellulose chain (Bajpai 2022). This is the classical model of polysaccharide degradation consists mainly of two categories of enzymes acting synergistically. The endo-acting enzymes (endo-glycoside hydrolases), and the exo-acting enzymes (exo-glycoside hydrolases) which hydrolyze the products of the former from chain ends. This model was generally accepted but a deficiency existed arising various questions. More specifically, it could not explain the way glycoside hydrolases were able to act on a polysaccharide chain in its environment. There were speculations for a substrate-disrupting factor that could make the substrate more accessible to the enzyme (Vaaje-Kolstad et al. 2010).

The answer to this problem was given by a discovered protein, CBP21, that increases substrate accessibility and the effectiveness of the hydrolytic enzymes. This protein was isolated from a microorganism that hydrolyzes chitin, a crystalline polymer analog of cellulose. These proteins were initially classified as carbohydrate-binding modules (CBMs) and belong to the family CBM33 as defined in the Carbohydrate Active Enzymes (CAZy) database (Vaaje-Kolstad et al. 2005). In detail, CBP21 can generate chain breaks in inaccessible and rigid parts of polysaccharides. It has to approach the chain from one side and then hydrolyze the well-ordered and solid structure of the substrate by a mechanism that leads to the oxidation of one of the new chain ends. The oxidation may enhance the disruption of the crystalline packing and the accessibility of the polysaccharide chain to cellulases. These proteins differ substantially from glycoside hydrolases. Similar to this protein family in Kingdom Fungi are the 61 glycoside hydrolases family that also act synergistically with cellulases. These families present structural

similarities. A diagnostic conserved arrangement of the N-terminal amino group and two histidines (His28/His19) that bind a metal ion. The mechanism of action of these enzymes remains unknown (Vaaje-Kolstad et al. 2010).

Later it was discovered that the members of the families CBM33 and family GH61 were lytic polysaccharide monooxygenases (LPMO) and a reclassification of these families took place. The families of lignin degradation enzymes and LPMO families were merged and a new CAZy class that was named auxiliary activity (AA) was launched (Levasseur et al. 2013). At present, there are three sequence-based families of these enzymes, namely the AA9 (formerly GH61), the AA10 (formerly CBM33), and the AA11 (Hemsworth et al. 2014). The structure of LPMOs is conserved, especially the histidine brace of the active site, which is the amino-terminal histidine-ligated mononuclear copper (Lo Leggio et al. 2015).

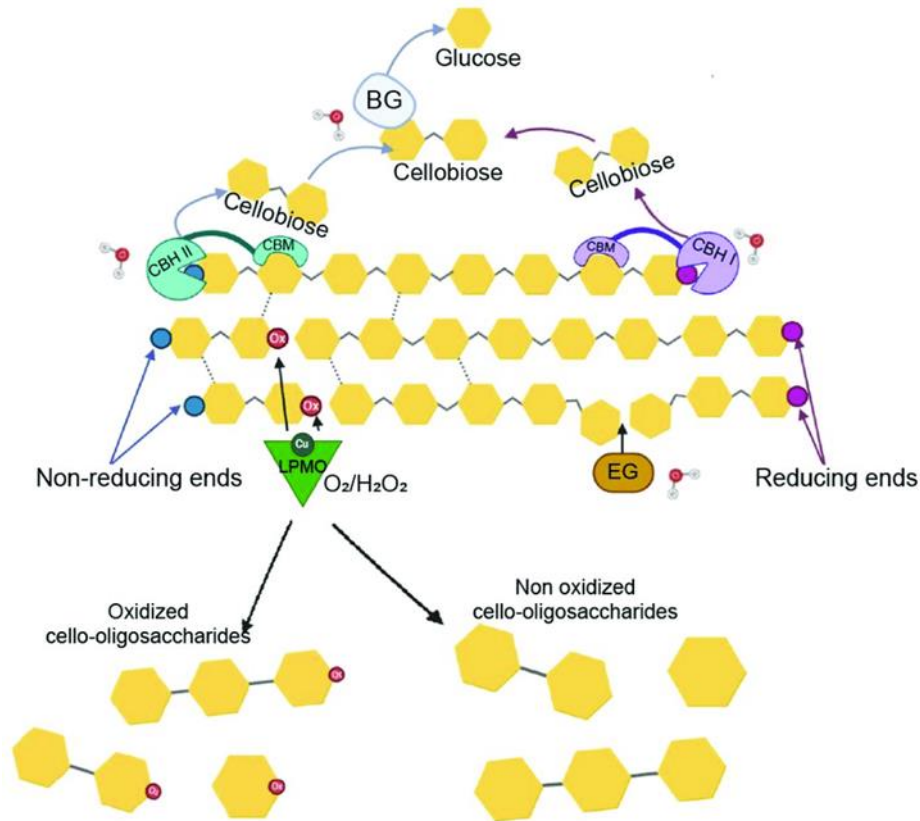


Figure 4. Enzymatic hydrolysis of cellulose (Calderaro, Bevers, and Berg 2021).

3. Cellulases

The simple composition and morphology of cellulose oppose the huge diversity of cellulase enzymes with catalytic modules categorized at 14 GH families. The 1,4- β -D-glucan-

cellobiohydrolases (CBH), the endo- β -D-glucanases (EGO), and the β -glucosidase (BG) are classified as cellulases. Their different substrate specificity allows them to act synergistically on cellulose. The CBHs or β -1,4-exoglucanases act usually processively on either the reducing or the nonreducing ends of a cellulose chain to liberate cellobiose as the primary product. The EGOs act more randomly hydrolyzing internal sites of the cellulose chain, producing oligosaccharides of various lengths. The BGs hydrolyze cellobiose, aryl-glucopyranosides, and cellodextrins to produce glucose (Xu et al. 2011). The majority of the cellulases are active at the temperature range of 20°C-60°C, and pH range of 4.0-8.0. However, there are some exceptions, isolated from extremophiles, whose activity and/or stability extends far beyond this range (Bajpai 2022).

4. β -1,4-exoglucanases or 1,4- β -D-glucan-cellobiohydrolases

The CBH degrades cellulose by hydrolyzing the 1,4- β -D-glycosidic bond, facilitating the production of primarily cellobiose. These enzymes usually belong to the glycoside hydrolases (GH) families 5, 6, 7, 9, 48, and 74. There are two types of CBH. The CBHI cleaves the cellulose chain progressively from the reducing, and the CBHII from the nonreducing end. Microcrystalline cellulose is degraded mostly by CBH, so these enzymes are of paramount importance to the hydrolysis of lignocellulose. Their catalytic domain features a tunnel-shaped structure that includes two surface loops covering the part of the active site. So CBH is equipped with the ability to attach to the ends of cellulose strands and then processively slide down the microfibrils cleaving off cellobiose as they progress. Their processive characteristics and the insolubility of the cellulose make their kinetics deviant from the Michaelis-Menten model (Bajpai 2022; Yeoman et al. 2010; Sinha, Sharma, and Yazdani 2023).

5. Glycoside Hydrolases

Glycoside hydrolases (GH) are a common group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety based on the CAZy database (Carbohydrate-Active enZymes Database, <http://www.cazy.org/>). There are more than 100 GH families, with 1621838 modules and about 44453 non-classified molecules. The classification of the GH into families is established on the amino acid sequence similarities and the relationship between sequence and folding similarities. This kind of classification allows the prediction of the structure and even the substrate specificity of enzymes for which only the amino acid sequence is known. These enzymes are usually nomenclature according to their substrate specificity and seldom to their molecular

mechanism. That kind of classification is independent of the structural features of these enzymes. Generally, the catalytic mechanism of the glycosidic bond hydrolysis is based on two amino acid residues of the enzyme, a general acid (proton donor), and a nucleophile/base. Depending on the spatial position of these catalytic residues, GH families are categorized into two classes: retaining or inverting. However, some GH families lack a catalytic nucleophile/base so they do not follow the typical catalytic mechanism. They replace it either with substrate-assisted catalysis, a network of several residues, the use of non-carboxyl residues, or exogenous nucleophiles. GH are very useful enzymes in the development of biofuels and disease research. They are also a part of the carbon cycle and allow microorganisms in the soil to decompose plant cells and release CO₂ aerobically as well as a variety of fermentation products anaerobically (Vuong and Wilson 2010). Many GH families are involved in the lignocellulose degradation. Specifically, the GH families 5, 6, 7, 8, 12, 45, and 48 which contain mostly fungal cellulases, the GH5, GH9, GH10, GH11, GH43, and GH48 that are found in cellulolytic bacteria (You et al. 2023).

6. GH48 FAMILY

The GH48 cellulases are one of the most abundant groups of glycoside hydrolases in nature. They are known to be expressed by more than 70 genera. According to the CAZy database (Carbohydrate-Active enZymes Database, <http://www.cazy.org/>), the GH48 family consists of chitinases, reducing end-acting cellobiohydrolases-exoglucanases, endo- β -1,4-glucanases, and cellobiohydrolases. The most abundant group in this family is the exocellulases that catalyze cellulose hydrolysis to release cellulose molecules. Only 18 enzymes of this family have been characterized and 11 have a reported crystal structure. In this family are included some endo-processive cellulases, which have a very similar activity to the cellobiohydrolases. The GH48 enzymes present a very characteristic 3D structure, which is the (a/a)₆ barrel. They present an inverting mechanism with aspartate and glutamate acting as base and acid donors.

Based on the NCBI data, there are about 201 GH48 enzymes translated by *Bacillus sp.*, but only 1 is biochemically characterized and 3 have a reported crystal structure or a predicted one. The GH48 is generally necessary for cellulose-degrading bacteria but they demonstrate intrinsically low activity. Their low activity is mainly attributed to end-product inhibition and the general properties of their substrates. These cellulases play an important part in the hydrolysis of crystalline cellulose. The genetic area that encodes these enzymes can act as a molecular marker

for cellulolytic activity since all organisms possessing at least one GH48 have proven to be truly cellulolytic organisms, especially in anaerobic environments. The detection and quantification of GH48 genes can be used for the identification of cellulolytic organisms. So these enzymes present also an important role in metatranscriptomics and genetic analyses.

The GH48 cellobiohydrolases initiate hydrolysis from either the reducing or nonreducing end of the cellulose chain. However, all the characterized ones are reducing end-acting cellobiohydrolases. The catalytic mechanism of these exoglucanases is depicted in Figure 5. More specifically, the substrate will slide into the enzyme's cleavage site (tunnel-shaped) and then the product will be released from the open shallow cleft at the exit. The catalysis is triggered by the occupation of +1 and +2 positions by the substrate that ends up in a conformational change of the (a/a)₆-barrel structure. This mechanism is a concerted, inverting, "substrate-control" mechanism and at least two carboxyl residues are required.

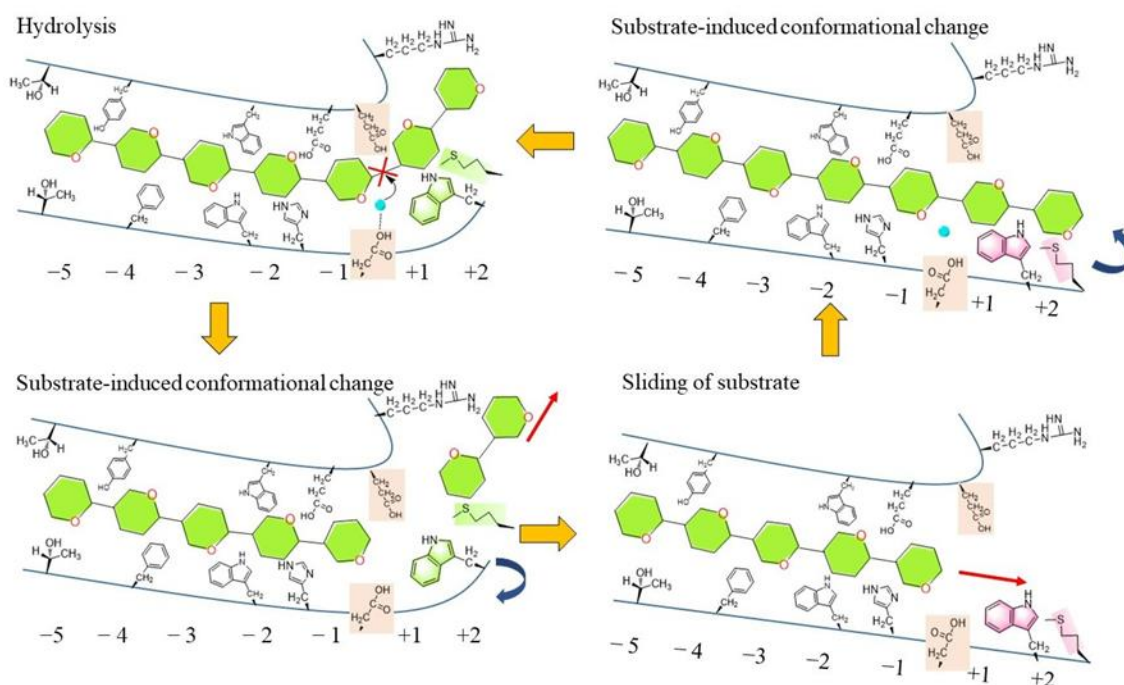


Figure 5. The hypothetical model of the processive action of GH48 cellulases (You et al. 2023).

The GH48 cellulases successfully synergize with the complementary endoglucanases either in free cellulase systems or cellulosome systems. The cellobiohydrolases of this family synergize with endoglucanases to enhance cellulose degradation efficiency and with β -glucosidases to eliminate feedback inhibition (You et al. 2023).

7. *Bacillus spp.*

The genus *Bacillus* belongs to the Firmicutes phylum and includes over 200 described species and subspecies. This genus was established by Cohn in 1872 (Soule 1932). About the general characteristics of *Bacillus spp.*, this genus has a rod-shaped morphology, they are Gram-positive bacteria, aerobic or facultatively anaerobic, and catalase-positive. One of their distinguishing characteristics is the formation of endospores (De Vos and Garrity 2009). Additionally, the nutritionally important metabolite, menaquinone 7 (MK-7), is synthesized by mainly using *B. subtilis* species (Singh, Puri, and Panda 2015). Because of their physiological ability and capability to form endospores, *Bacillus spp.* presents resistance to various environmental conditions and omnipresence in adverse habitats, like soil or intestinal flora. They are among the most widespread endophytic bacteria. They also can be easily formulated and stored thanks to their ability for endospore formation. The *Bacillus spp.* includes mainly non-pathogenic but also pathogenic species. Most of them and their products are believed to be safe for intended environmental use. This genus consists of species that are investigated as biocontrol agents. Their antimicrobial activity is mainly based on the production of hydrolytic enzymes such as chitinases, chitosanases, glucanases, cellulases, lipases, and proteases, which efficiently hydrolyze the major components of the fungal and bacterial cell walls (Miljaković, Marinković, and Balešević-Tubić 2020).

8. *Bacillus safensis*

Bacillus safensis was isolated from spacecraft surfaces (Mars Odyssey Orbiter) and assembly facility surfaces at the Jet Propulsion Laboratory in California and the Kennedy Center in Florida. The “safensis” was derived from where it was first isolated, SAF (the spacecraft-assembly facility). The members of this species are mesophilic, aerobic, chemoheterotrophic, Gram-positive, spore-forming rods that are motile using polar flagella. The first thirteen strains that were isolated indicated a 99.9% sequence similarity with *Bacillus pumilus* but DNA–DNA hybridization showed only 54–66% DNA relatedness between them and MALDI-TOF MS analyses distinguished the two species (Satomi, La Duc, and Venkateswaran 2006). This species is closely related to *Bacillus pumilus*, *Bacillus altitudinis*, *Bacillus xiamenensis*, and *Bacillus invictae*. *B. safensis* has been erroneously identified as *B. pumilus*, especially when methods with high sensitivity did not take place. This species has potential for biotechnology applications because of its great variety of industrial enzymes and secondary metabolites. *Bacillus safensis* colonizes a

wide range of habitats, many of which are stringent for the survival of some microorganisms. It also presents a few plant growth-promoting traits and can colonize habitats that usually are hostile to microbial growth. It is regarded to be a safe industrial microorganism (Lateef, Adelere, and Gueguim-Kana 2015).

9. Applications of exo-glycosidases

Cellulose enzymes have several applications including textile, paper and pulp, food, animal feed, fuel, and chemical industries. It is also used in waste management, pharmaceutical industry, protoplast fusion, and genetic engineering. Although, exo-glycosidases are not present in all of the above applications. Some of the applications that exo-glycosidases are used are food, beverage, animal industries, and biofuels, which are mentioned more extensively below.

Food Industry

Population increase, urbanization, and climate change are destructive influences on food production. The main goal of the food industry is the process of food to retain or improve its nutritional qualities, eliminate human health threats, and extend the product's shelf-life. There are also endeavors to improve food's flavor, colour, texture, or convenience to pack and consume. Cellulases are used to increase the nutritional properties of the products. They can also affect the baking process when they are in their pure form. Thus, cellulases are used in the baking industry to break up roughage in dough. They are usually combined with pectinases and hemicellulases in the food industry. These three enzymes are used for olive oil extraction. They improve the oil's extraction, quality, and antioxidants, and they ensure a lower chance of rancidity and less wastage. Cellulases can aid in the extraction of carotenoids from vegetables like tomatoes. Carotenoids can be a part of food processing as coloring agents in both food and beverage industries (Kumar V et al. 2019; Ejaz, Sohail, and Ghanemi 2021).

Beverage Industry

The Beverage industry is the largest food processing industry and a very profitable one. It includes both alcoholic and non-alcoholic drinks. It uses fermentation to produce plant-based and animal-based beverages. Cellulosic biomaterials are perfect for alcohol production since 90% of ethanol is produced by natural sugars and starch. Cellulose and hemicellulose are the main source of sugars. Cellulases can provide good-quality alcohol with better properties even from lower-quality raw materials. The combination of cellulases, pectinases, and xylanases can

improve the extraction and filtration rate in winemaking. They have a lower energy demand for fermenters and they result in better wine colour extraction, skin maceration, quality, and stability with lower viscosity. In beer brewing, it has been observed that two cellulases, an endoglucanase I and an exoglucanase II of the *Trichoderma* cellulase system, can result in a maximum reduction in the degree of polymerization and wort viscosity. Regarding non-alcoholic drinks, the application of cellulases in juice manufacturing can increase their extraction, clarification, and stabilization. They hydrolyze cellulose fibrils, making the filtration of juices easier. It can also solubilize the cell wall polysaccharides leading to almost complete liquefaction of the fruit pulp after crushing the fruit. Cellulases improve the taste, texture, and aroma and decrease the bitterness in citrus fruits (Kumar V et al. 2019; Kuhad, Gupta, and Singh 2011).

Feed Industry

The available commercial feeds are not able to satisfy the nutrient requirements of animals in animal farms, and more than half expenses concern their food. Cellulose is the primary food for many organisms but not all mammals have a cellulose-digesting ability. The lignocellulosic materials are poor in protein and digestibility coefficients but rich in fibers and anti-nutritional factors. Cellulases and other enzymes are used to increase the digestibility of these materials. They also degrade the anti-nutritional factors, improving the nutritional value of these materials, and thus animal health and performance. The process of animal feedstock production usually includes heat treatment. The usage of thermophilic cellulases aids in the reduction of pathogens and the enhancement of the digestibility and nutrition of the feed. Their antimicrobial action assists in the reduction of ionophore antibiotic usage (Kuhad, Gupta, and Singh 2011; Sharada et al. 2014).

Biofuels

Technological innovations worldwide are blooming because of the efforts for the reduction of carbon emissions. There is a global attempt to replace petroleum-derived products with bioproducts and biofuels from renewable plant biomass. The inedible lignocellulosic biomass is rich in convertible carbohydrates and aromatics for this purpose (Glasgow et al. 2021). Biofuels can be effortlessly produced by local resources and may be the most feasible option for the reduction of carbon dioxide by the transport section. Second-generation biofuels are more difficult to be produced than the first-generation ones and they are more expensive on an

energy-equivalent basis (Bajpai 2022). Bioconversion of renewable lignocellulosic materials into useful and higher-value products (biofuel and other byproducts) is perhaps the most popular application of cellulases. There are three steps in the degradation of renewable lignocellulosic biomass to be used in biofuel production, the pretreatment of biomass, the saccharification, and the fermentation. Cellulases and other enzymes are involved in the second step where bioconversion occurs. Cellulases' products from hydrolysis of this substrate are simple sugars, pentoses, or hexoses. These sugars are converted to fuel or bioethanol at the final step (Ejaz, Sohail, and Ghanemi 2021). Enzymatic hydrolysis decreases the utility cost compared to chemical hydrolysis as it is usually conducted at mild pH and temperature conditions and does not have corrosion issues. Some factors have a negative effect on this hydrolysis procedure, like end-product inhibition, thermal deactivation of the native protein, nonspecific binding to lignin, and irreversible adsorption of the enzymes to the heterogeneous substrate. Thus, two goals must be achieved to make the use of enzyme hydrolysis the most profitable one: (1) optimizing the enzyme production, and (2) optimizing the catalysis system that is based on cellulases. Multiple solutions have been developed to achieve these goals and they are still an active research field (Kuhad, Gupta, and Singh 2011).

MATERIALS AND METHODS

1. Instruments and Devices

Instruments and Devices	
Photometer Multiskan GO	Thermo Scientific, USA
Photometer Hitachi UV 2000	
PCR machine TC-1000-S	Drawell, China
Protein electrophoresis chambers	Bio-Rad, USA
pH meter 537	WTW, Germany
Magnetic stirrer Orbit LS	Labnet, US
Autoclave	Macherey-Nagel, Germany
BOECO Thermo-shaker for Microtubes TS-100	Hamburg, Germany
Thermostatic Water Bath	
Hotplate Stirrers	
Centrifuge TJ-6	Counter, USA
Refrigerated Ultracentrifuge	
Microcentrifuge Eppendorf 3200	Germany
High-performance anion-exchange Chromatography with pulsed amperometric detection (HPAEC-PAD)	Thermo Scientific

Ultrasonic Processor	Sonics & Materials Inc, Newtown, USA
Protino Ni-NTA Columns 14mL	Macherey-Nagel
Ultrafiltration spin columns 6 MWCO 30,000	Vivaspin™
Protein Concentrators Pierce™ PES 6mL, 30K MWCO	Thermo Scientific
Sephadex G25M PD10 column	CYTIVA, Life Sciences, Manassas, VA, USA

2. Reagents and Solutions

Reagents and Solutions				
Buffer solutions				
Phosphate Buffers	Na2HPO4 100mM		NaH2PO4 50mM	pH=6.0
	Na2HPO4 100mM		NaH2PO4 50mM	pH=7.0
	Na2HPO4 500mM		NaH2PO4 500mM	pH=8.0
TGS Buffer	(10x Tris-glycine-SDS)			
Solutions				
Imidazole	1.5M C3H4N2			
Sodium chloride	5M NaCl			
50x TAE	242 g Tris Base, 57.1 mL Glacial acetic acid, 100 mL of 0.5 M EDTA, pH 8.0			
Acrylamide/Bisacrylamide	30% acrylamide (C3H5NO)		0,8% bisacrylamide (C7H10N2O2)	
NPI10	50mM NaH2PO4	300mM NaCl	10mM Imidazole	pH= 8.0
NPI250	50mM NaH2PO4	300mM NaCl	250mM Imidazole	pH= 8.0
NPI500	50mM NaH2PO4	500mM NaCl	10mM Imidazole	pH= 8.0
Reagents				
Bradford reagent	100 mg/L Coomassie Brilliant Blue		50ml/L C2H5O	100ml/L H3PO4
D-Glucose Assay Kit	Biosis S.A.			
Other materials				
1kb DNA Ladder (BioLabs)				
Gel Loading Dye 6x (Bio Labs)				
Pre-Stained SDS Page Protein Ladder (FastGene)				
Luria Bertani (LB) medium				
Use was made of analytical grade chemical reagents from the companies Sigma (USA), Merck (Germany) and CIBA (Switzerland).				

2.1. Regenerated amorphous cellulose (PASC) preparation

PASC was prepared at 2% w/v concentration following a modified version of the protocol published by Zhang et al. (2006). Briefly, 1 gr of microcrystalline cellulose (FMC PH-105) was weighed and added to a flask and 3 mL of distilled water (dH₂O) was added to hydrate the powder and create a cellulose-suspended slurry. In this slurry, 50 mL of 85% ice-cold phosphoric

acid (H₃PO₄) solution was slowly added under vigorous stirring. The sample was incubated for 1 hour with continuous stirring and then 200 mL of ice-cold dH₂O were slowly added. The precipitated cellulose was centrifuged at 4000 rpm for 20 min. The pellet was suspended by ice-cold water, followed by centrifugation to remove the supernatant containing phosphoric acid four times. Approximately 0.1 mL of 2 M Na₂CO₃ was added to neutralize the residual phosphoric acid, and then, 45 mL of ice-cold distilled water was used to suspend the cellulose pellet. After centrifugation, the pellet was suspended by distilled water and centrifuged twice or until pH 5.0-7.0 (Zhang et al. 2006).

3. Microorganisms and strains

- *Bacillus safensis* (ATHUBA63)

This microorganism was isolated from soil samples from Athens, Greece. Originally this strain was characterized as *Bacillus pumilus* (Paul Christakopoulos et al. 1999). These two species have a great similarity in their 16S rRNA gene sequence.

- *Escherichia coli* DH5a

These are competent cells for routine cloning. This strain is utilized for small plasmid propagation and is selected for its ability to achieve high transformation efficiency.

- *Escherichia coli* BL21 (DE3)

These are competent cells ideal for routine T7 expression. This strain is suitable for the expression of non-toxic heterologous genes with IPTG induction.

4. Bioinformatic analysis

A bioinformatics analysis took place at the beginning of this research to locate the genome segment that expresses the BsGH48 from a *Bacillus safensis* strain. The available gene sequences from *gh48* cellulase genes of this and related species were aligned, using Clustal Omega multiple alignment online tool (<https://www.ebi.ac.uk/Tools/msa/clustalo>). Based on this alignment, primers were constructed and the *bsgh48* was located. The genome segment was examined for the existence of signal peptide sequences with an online page <https://services.healthtech.dtu.dk/services/SignalP-5.0/>. The signal peptide is usually excluded from the final genome segment that will be used in the transformation procedure. To translate the DNA sequencing to amino acid sequence and indicate the gene's translation starting point

the online tool <https://web.expasy.org/translate/> was used. Finally, SnapGene was used to demonstrate the genomic sequence and therefore to choose the appropriate restriction enzymes for the ligation procedure and to design the primers.

5. Protein expression and Purification

5.1. Amplification and isolation of the genome segment that expresses BsGH48 from a *Bacillus safensis* strain

5.1.1. Culture of *Bacillus safensis* on nutrient agar

Bacillus safensis was cultured on a Luria Bertani (LB) agar plate (tryptone 1%, yeast extract 0,5%, NaCl 1%, agar 1,5% by weight). After the preparation of the medium, the pH was adjusted to 7.0 using a 5M NaOH solution. Then, the LB agar medium was autoclaved. The microorganism was plated using the Streak Plate Technique to isolate single colonies.

5.1.2. Colony Polymerase Chain Reaction (PCR)

The amplification of the genome segment that expresses BsGH48 was performed by Polymerase Chain Reaction (Colony PCR) using a single colony of the microorganism from the LB agar plate and the primers listed in Table 1. This technique was carried out using a thermal cycler (Gradient PCR Thermal Cycler-DRAWELL) and sterile 0.5 mL PCR tubes (Scientific Specialties Inc.). The final reaction volume was 20 µL, which consisted of 18 µL PCR Master mix (the estimated ratio of which is 0.43 µL dNTPs, 3.4 µL Phusion Buffer, 1.7 µL Phusion hot start DNA polymerase (2 U/µL)) and 2 µL forward and reverse primers (10 pmol/µL). The Polymerase Chain Reaction steps that took place were as follows: "initial denaturation" 2 min, 98°C; 24 cycles of "denaturation" 30 s, 98°C; "annealing" 30 s, 59-62°C; "extension" 1.5 min, 72°C; "final extension" 5 min, 72°C; preservation of the samples at 12°C.

Table 1. The primers for the bsgh48 gene isolation.

Primers	Length (bp)	Tm (°C)
GH48-ATG-NotI-F		
5'-GCGCGCGGCCGC <u>ATGTCAAACAAAGAACGGTTTTTAACG</u> -3'	27	61
GH48-TTG-NotI-F		
5'-GCGCGCGGCCGC <u>TTGTCAAACAAAGAACGGTTTTTAACG</u> -3'	27	61
GH48-SpeI-R		
5'-GCGCACTAGTTTAGTTGATCAGTCGATGATATTCAGC-3'	27	60

5.1.3. Electrophoresis of PCR products

Agarose gel (1% w/v) was used for electrophoretic separation of the Polymerase Chain Reaction products. For the preparation of this gel, 1.5 g of agarose was weighed and then added to a solution of 1× TAE volume 150 mL (which results from the dilution of 50× TAE). To solubilize the agarose, the mixture was heated in a microwave oven (850 Watts). After its homogenization, the solution was brought back to room temperature, and 3.7 µL of EtBr was carefully added to it. Finally, the solution was poured into an electrophoresis vessel to solidify. The electrophoresis was carried out at 100 Volt, for 30 min.

5.1.4. DNA isolation from Agarose gel

The bacterial DNA was isolated from the Agarose gel using the "Gel and PCR Clean-up" kit (NucleoSpin®), following the company's recommended protocol. Firstly, the desired part of the gel was cut off under an ultraviolet (UV) lamp. The isolated DNA was stored at -20°C.

5.1.5. DNA quantification

The quantification of the isolated DNA was performed by measuring it with a Nanodrop-Microplate Photometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer), at 260 and 280 nm. The sample (2 µL) was diluted ten times with dH₂O and then the absorbance was measured at 260 and 280 nm. The blank was the isolation solution from the kit diluted accordingly.

5.2. Amplification and isolation of the plasmid cloning vector

5.2.1. Amplification of the vector

The plasmid vector used in this research is pet15b16s, a vector based on the pET-15b plasmid (Addgene), modified on its multi-cloning site to incorporate the *NotI* and *SpeI* restriction sites (Glekas et al. 2022). It was introduced into *Escherichia coli* DH5a cells to be enhanced by way of the process called transformation. Approximately 0.2 µL of isolated plasmid vector (depending on its concentration in the solution) was added to 300 µL of receptive cells. The heat shock procedure was followed to introduce the plasmid vector into the bacterial cell. That is, the sample was heated to 42°C for 1.5 min and placed on ice for 5 min. These steps were repeated in duplicate. Next, 900 µL of LB medium (tryptone 1%; yeast extract 0.5%; sodium chloride 1%; pH 7) was added to the sample. The 1.5 mL Eppendorf tube containing the sample was sealed using parafilm (to avoid contamination) and incubated at 37°C, with continuous agitation, for 1 h. After incubation, the sample was centrifuged at 2000 rpm for 5 min, 900 µL of supernatant

was removed in a laminar airflow chamber and the pellet was re-dissolved in the remaining 100 μ L. Finally, the sample was plated on LB Agar, containing 0.1 mL of ampicillin solution (100 μ g/mL) per 100 mL of LB agar. Bacterial cells that have received the plasmid vector, will only grow, as this vector has an ampicillin resistance gene. The plate was incubated at 37°C for 16 h (overnight).

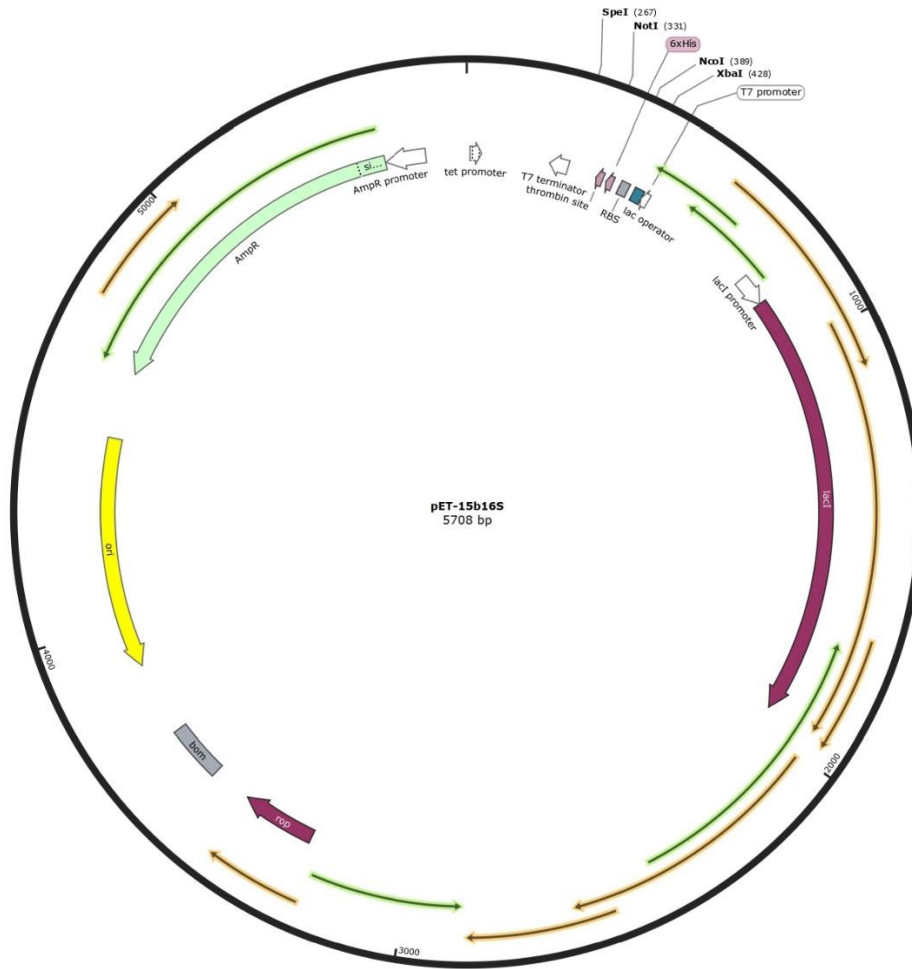


Figure 6. The pet15b16s plasmid vector was depicted by SnapGene.

5.2.2. Isolation of the vector

The isolation of the amplified plasmid vector was carried out by inoculating single colonies into an LB liquid medium containing ampicillin. Cultures were conducted in 100 mL Erlenmeyer flasks, containing 20 mL of LB liquid medium (ratio of the medium's volume to the total volume of the flask 1/5 v/v). Following inoculation, the flasks were incubated in an orbital shaker, operating at 37°C and 180 rpm for 16 h (overnight). At the end of the incubation period, the

optical density was measured at 600 nm (OPTIZEN™ POP Spectrophotometer). For the isolation of the amplified plasmid vector, the recommended protocol "Isolation of High-Copy DNA Plasmid from *E. coli*" of the company (NucleoSpin®) was followed using the appropriate kit. Finally, the isolated and amplified plasmid vector was stored at -20°C.

5.3. Recombinant Plasmid Construction (Recombinant Cloning)

The recombination of the plasmid vector (pet15b16s) with the gene (*bsgh48*) was carried out using the restriction enzymes *NotI* and *SpeI*. The pet15b16s vector and *bsgh48* were incubated separately with the restriction enzymes at 37°C, overnight. The final volume of the reaction with the vector was 20 µL and with the gene was 50 µL. Afterward, the product of the recombination enzymes was cleaned with an agarose gel electrophoresis from the DNA that was not digested. The digestion products were isolated from the agarose gel and quantified. For the insertion of the gene of interest into the plasmid cloning vector, a ligase is used. According to literature, the ratio of the digested plasmid vector to the gene should be 1/3. However, the following formula can be used for the calculation of the insert:

$$ng\ insert = 100ng\ vector \times \frac{the\ size\ of\ the\ insert}{the\ size\ of\ the\ vector}$$

The ligation mixture had a final volume of 10 µL and was incubated for 2h at 25°C or 4°C, overnight. Next, the recombinant vector must be amplified as mentioned in the section "5.2.1. Amplification of the vector".

5.4. Genetic modification of *Escherichia coli* BL21 (DE3) cells

The high-copy recombinant vector was isolated from the DH5a *E. coli* strain and it was quantified. The recombinant vector was then inserted in the BL21 (DE3) *E. coli* strain, following the same protocol as for DH5a.

5.5. Overexpression of BsGH48

The overexpression of the BsGH48 started by inoculating a single colony of the transformed BL21 (DE3) *E. coli* strain into 20 mL LB broth, containing 20 µL of ampicillin (100 µg·mL⁻¹), which was incubated at 37°C, overnight. This culture served as a preculture for the scale-up cultures, which were conducted in 3L Erlenmeyer flasks containing 600 mL LB broth and 600 µL of ampicillin (100 µg·mL⁻¹). A specific volume of the preculture (so that the initial optical density of the cultures will be 0.05), was used to inoculate the cultures. Following inoculation, cultures

were incubated in an orbital shaker operating at 37°C and 180 rpm, until the optical density reached approximately 0.5-0.6. Then 100 µg·mL⁻¹ IPTG (Isopropyl β-D-1-thiogalactopyranoside) were added and the cultures were incubated at 18°C, overnight. The above-described method was called “Overnight Batch”. Another overexpression method was tested, called “Fast Batch” method. The procedure was the same as described above except that after the addition of IPTG incubation was conducted at 37°C for 3 h.

5.6. Isolation and purification of BsGH48

Cells were harvested by centrifugation at 4000 rpm for 20 min at 6°C. The cell pellet was resuspended in equilibration buffer NPI10 and sonicated. The duration of the sonication was 30 min and every 9 secs were followed by 3 sec of ultrasound. The samples were centrifuged at 4000 rpm for 20 min, and the supernatant was collected. The enzyme was purified by affinity chromatography using a Ni-NTA column (Qiagen, Hilden, Germany) following the manufacturer’s protocols. Ni-NTA column was initially eluted with, 20 mL of NPI10 buffer followed by 35 mL of NPI250 buffer, and finally 10 mL of NPI500 buffer (Figure 7). The BsGH48 was eluted with the buffer NPI250. The preparation and the content of the solutions are listed in the Table “Reagents and Solutions”**Error! Reference source not found..**

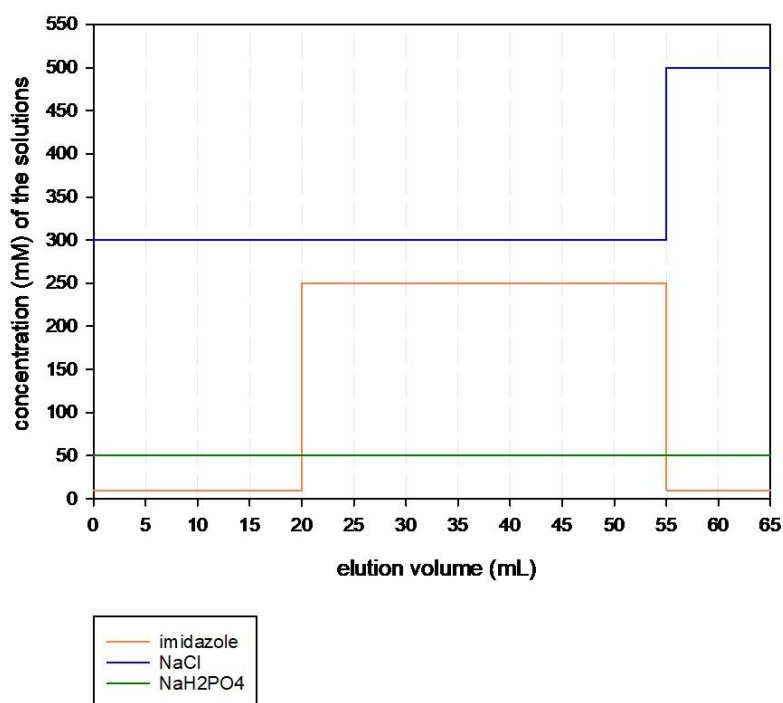


Figure 7. Solutions gradient to the affinity chromatography.

Finally, gel filtration was conducted using a Sephadex G25M PD10 column (CYTIVA, Life Sciences, Manassas, VA, USA), to desalt the sample, and exchange the buffer to 0.1 M Phosphate Buffer, pH 7.0.

5.7. SDS-PAGE

A Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was conducted to determine the fraction containing the BsGH48 and to assess its purity (Laemmli 1970). A 10% acrylamide gel was used, the composition of which is listed in Table 2.

Table 2. Content of the acrylamide gel.

Acrylamide gel SDS-PAGE (10%)		
mL	Separating gel 10%	Stacking gel
dH ₂ O	4.09	3.023
Acry/Bis	3.3	0.667
Lower Tris (1.5 M)	2.5	-
Upper Tris (1 M, pH 6.8)	-	1.25
20% w/v SDS	0.05	0.025
10% w/v APS	0.05	0.025
Temed	0.01	0.01
Final volume	10	5

5.8. Concentration of BsGH48

After the purification of the protein molecule, an ultrafiltration membrane spin column was used to achieve rapid concentration. These columns combine the principle of size exclusion with centrifugation. For this experimental procedure, the column that was used had a molecular weight cut-off (MWCO) value of Mr 30.000 and it was centrifuged at 10.000 rpm repeatedly until the desired final volume of the protein sample.

5.9. Protein determination

The Bradford assay was conducted for the quantification of the protein. This assay is based on the shift in absorbance maximum of Coomassie Brilliant Blue G-250 dye from 465 to 595 nm following binding to denatured proteins in solution (Bradford 1976). 10 µL of the protein sample and 10 µL of a blank sample were added in 200 µL Coomassie Brilliant Blue G-250 dye each, in a 96-well plate. The plate was incubated at room temperature for 5 min, without being exposed to any light source, and finally, the absorbance at 595 nm was measured. Quantification of the protein was achieved using the appropriate standard curve.

6. Characterization of BsGH48

6.1. Activity Determination of BsGH48

Exo-cellulase activity was determined using PASC as substrate. The reaction consisted of 50 μL of properly diluted enzyme and 250 μL of 1% w/v PASC in 100 mM citrate-phosphate buffer (pH 5.0). Incubation was conducted in a thermoshaker operating at 1000 rpm and 50 °C for 60 min. Following cooling of the samples in an ice bath, the reducing sugars were measured using the DNS method (Miller 1959). The DNS reagent was added and the samples were centrifuged for 10 min at 12.000 rpm, to remove the insoluble substrate. The supernatants were collected, boiled for 5 min, and the absorbance at 540 nm was measured. The concentration of the reducing sugars was estimated using a glucose standard curve.

6.2. Biochemical Characterization of BsGH48

6.2.1. Optimum pH

The optimum pH of BsGH48 was determined using the standard assay, modifying the time (180 min) and the temperature (45 °C), using the buffer systems presented in Table 3.

Table 3. Buffer systems for the optimum pH determination.

Buffer systems for the optimum pH determination	
100 mM	
Buffers	pH range
Citrate Phosphate Buffer	3.0-5.0
Phosphate Buffer	5.0-8.0
Tris-HCl Buffer	8.0-9.0

6.2.2. Optimum temperature

The optimum temperature was determined using the same assay as for the estimation of optimum pH. The temperatures tested ranged from 20-90 °C.

6.2.3. Effect of various Modulators

The effect of various modulators on the BsGH48 activity, like metal ions (Na^+ , K^+ , Cu^{2+} , Ca^{2+} , Ba^{2+} , Mn^{2+} , Zn^{2+} , Mg^{2+} , Co^{2+} , Fe^{2+} , and Fe^{3+}), SDS (anionic surfactant) and EDTA (chelator) was investigated. The concentration of the solutions containing these modulators was 5 mM. The enzyme was pre-incubated with the modulators at room temperature (25 °C) for 30 and 60 min. The activity of the enzyme was measured by conducting the standard assay. The enzyme activity in the absence of modulators was considered to be 100%.

6.2.4. Substrate specificity

Substrate specificity of BsGH48 was examined toward PASC, Avicel, CMC, Lichenan, β -Glucan, 2NP- β -D-Galactopyranoside, pNP- α -D-Galactopyranoside, pNP- α -D-Glucopyranoside, pNP- β -D-Glucopyranoside, 4NP- β -D-Xylopyranoside, A-cellulose, XYLAN-Oat spell, Arabinoxylan, Xylan Birchwood, and Xylan Beechwood. All the substrates were diluted in 100 mM, pH 5.0 citrate-phosphate buffer. The activity of the enzyme was measured by conducting the standard assay.

6.2.5. Kinetic constants

The kinetic constants, which are V_{max} , and K_m were ascertained by conducting the standard assay and a range of concentrations of PASC (0.2-1% w/v) and Avicel (1-5% w/v). The data obtained were fitted to the Michaelis–Menten model.

6.3. Stability of BsGH48

Both the pH and thermal stability of this enzyme were studied. To determine the pH stability of BsGH48, the enzyme was diluted in a set of buffer solutions (Table 3) and incubated at 4 °C for 24 hours. The residual enzyme activity was measured by conducting the standard assay. The thermal stability of BsGH48 was tested by incubated the enzyme at different temperatures (40, 50, 60, 65, and 70 °C). Aliquots were withdrawn at different time intervals and were cooled immediately in an ice bath. Their residual enzymatic activity was determined as the pH stability test.

6.4. Processivity assay

To determine the BsGH48 processivity the standard assay was carried out. The samples were cooled immediately in an ice bath and centrifuged at 13000 rpm for 10 min. The amount of reducing sugars was estimated in the supernatant using the DNS method. The solid residue was washed with 100mM citrate-phosphate buffer pH 5.0 twice. Then, the amount of insoluble reducing ends in rinsed PASC was measured, using the DNS method (Miller 1959).

6.5. Synergism of BsGH48 with BsGH5 and BSGH9

The ability of three different enzymes to hydrolyze the PASC, as the products that occur after the reaction were studied. Then, the same experiments were carried out for different combinations of these three enzymes, to discover their synergistic action. These enzymes are BsGH5, BsGH9, Bs48. They were all from the same origin as BsGH48. 50 μ L of the properly diluted enzyme was added in 250 μ L of PASC 1% w/v, 300 μ L final volume. Both, the enzyme and

the substrate (2% w/v the original concentration of PASC) were diluted with 100mM phosphate buffer pH 5.5. The reactions were incubated in thermoshakers at 55 °C with 1000rpm for 0, 2, 4, 6, 8, 10, and 24 hours. By the end of the enzyme reaction, the samples were centrifuged at 12.000 for 10 min. The supernatants were collected, diluted with ultrapure water, and then filtered (0.22 µm). The dilution depends on the concentration of reducing sugars in the supernatants providing that the measurements are within the detection limits of the method. Usually, the samples were diluted ten times. The samples were analyzed for glucose (G1) to cellohexaose (G6), the products of the enzymatic reaction, through high-performance anion-exchange chromatography (HPAEC). Blanks were analyzed in equivalently treated reaction mixtures but without enzyme addition, because of the background noise created by PASC. The HPAEC system that was used consists of a CarboPac PA-1 (4 × 250 mm, Dionex) column and a pulsed amperometric detector (PAD) equipped with a gold electrode. The analysis was conducted with a gradient exchange of two solvents at a flow rate of 1 mL/min. The solvents were NaOH 100 mM (solvent A) and 1 M NaOAc in 100 mM NaOH (solvent B) and the protocol of this exchange was the following: : 0–10 min 0–10% B, 10–25 min 10–30% B, 25–30 min 30–100% B 30–30.2 min 100–0% B, 30.2–39.2 min isocratic step, 100% A. For the quantification of the products, the appropriate standard curves were created by the use of glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6), that consist of cello-oligosaccharides (COS) standards (Megazyme, Ireland).

RESULTS

1. Bioinformatic analysis of the gene cassette, coding the cellulolytic enzymes, of the natural strain *Bacillus safensis*

The location of the genome segment that expresses the protein of interest from the *Bacillus safensis* strain and the designing of the primers was conducted by the research team of the Microbial Biotechnology Department of Biology National and Kapodistrian University of Athens. It must be mentioned the fact that BsGH48 did not have a signal peptide, so it is not an extracellular protein.

After sequencing the genome segment that expresses the protein of interest from a *B. safensis* strain, the result was the following:

> BsGH48 Sequencing Protein

GATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCTCTAGAAATAA
TTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCC
TGGTGCCGCGCGGCAGCGGCCGCATGTCAAACAAAGAACGGTTTTTAACGCTTTATCATCAAATTTAAAG
CGATGCGAATGGATATTTTACCAGAAGGAATCCGTATCATTCATCGAGACGCTCATTTGTGAAGCA
CCTGATTATGGGCATATGACAACTTCAGAAGCGTATAGCTATTGGCTGTGGCTTGAGGTGCTGTACGGAC
ATTACACAGGGGACTGGAGCAAATTAGAGGTAGCTTGGGACAATATGGAGAAGTACATCATTCAGTAA
ATGAGGATGGACATGATGAGCAGCCGCATATGAATGCTTATAATCCGTCCAGTCCAGCCACATACGCAG
CAGAGAAACCATTTCCGGATCAATACCCAAGCCAATTAACCGGTGCACGTTCTGCTGGTCAAGATCCAAT
TGATAGTGAATTGAAATCAACCTATGGATCGAATGAAACGTATTTAATGCACTGGCTCCTTGACGTAGAT
AACTGGTATGGCTATGGAACTTGCTCAACCCATCACATAAAGCGGCATATGTGAACACCTTCAGCGTG
GTCCACAGGAATCTGTATGGGAGTCCATCCCTCATCCATCACAGGACGATAAATCTTTTGGCAAGCCGAA
TGAAGGCTTTATGAGCTTGTTCAAAAAGAAAATCAAGCTCCAGCTGCTCAGTGGCGCTATACGAATGCA
ACGGATGCTGATGCACGTGCGATCCAAGCGATGTATTGGGCAAAGGAGCTCGGCTATAATAAGCCGGCT
TATCTTGATAAAGCGAAAAAATGGGGGATTTCTCCGTTACGGCATGTACGATAAATATTTTCAAACGA
TTGGAAGCGGGAAGCAGGGGAATCCGTATCCTGGAAACGGAAAGGGCGGCTGTCATTATTTAATGGCA
TGGTATACCTCTTGGGGCGGAGGACTTGGGGAGTATGCCAACTGGTCATGGCGTATCGGARCGAGCCAT
TGTCATCAGGGATATCAAAATCCTGTCGCCGTTATGCTTTATCCACAGATAAAGGAGGCTTAAAGCCAT
CTTCAGCTACTGGGGCAAGCGATTGGGAAAAAACATTAAAGAGGCAGCTTGAATTTTACGTATGGCTTCA
ATCGAAAGAAGGAGCCATTGCAGGAGGCGCAACCAATAGCTGGAATGGAGACTATAGCGCTTATCCGG
CGGGAAGAAGTACTTTTTACGATATGGCATATGAAGATGCGCCTGTCTACCTTGATCCGCCATCAAATAA
CTGGTTTGGGATGCAGGCGTGGCCAATGGAGCGGTTGCAGAGCTTTATTATATTTTGTCAAAGATGGC
GACAAAACATCTGAAAATGTCCAAATGGCCAAATCTGCTATAACAAAATGGGTCAACTATGCATTAGATT
ACATTTTTATCGGCAGCCGTCCTGTGTCGGATGAAGAGGGTTATTTCTTAGATGACCAAGGACAAAGAAT
TCTAGGGGGAACAAACGTTTCTGTTGCTACAACAAGTGCGCCTGGAGAGTTTTGGCTTCTGAAAATATT
GCTTGGAGCGGACAGCCTGATACTTGAATGGGTTCCAGTCTGCAACAGGAAATCCAAATTTAACAGCT
GTAACAAAAGATCCGACTCAGGATACGGGCGTTTTAGGTAGTCTTGCCAAAGCTTTGATATTCTTTGCTG
CGGCCACAAAATGGAACAGGTGATTATACGGCGCTTGGTGCAAGAGCGAAAGATGCAGCCGCGCAG
CTCCTAGAGGTTGCATGGAATTACAATGATGGCGTCCGCATAGTGACAGAGGAAGATAGAGAAGACTAT
GACCGTTTCTTTAGAAAAGAAGTGTATTTCCCAATGGATGGAACGGGACATTTGGTCAAGGAAATCAG
ATTCTGGAGCCAGCACCATTCTTCTGACCCGAGCGAGGAGGGAATGGGGTCTATACGATTTTGTCTG
ATCTTCGTCCGAAGATTAAACAAGATCCAGCGTGGTCTTCACTCGAAAGTAAATATCAGTCTTCATTTAAT
GAAGCTACAGGCAAATGGGAGAATGGCGCACCAAGTTTTCACTTATCACCGCTTCTGGTCTCAAGTGGATA
TGGCCACTGCCTATGCTGAATATCATCGACTGATCAACTAACTAGTCAATAAAGTAGCATAACCCCTTGGG
GCCTCTAAACGGGTCTTGAGGGGTTTTTGTCTGAAA

The functional non-coding regions of the gene are marked with different colours and their functions are described in Table 4 and depicted in Figure 8.

Table 4. Functional non-coding regions of the *bsgh48*.

Functional non-coding regions of the <i>bsgh48</i>	
TAATACGACTCACTATAGG	T7 Promoter
GGAATTGTGAGCGGATAACAATTCC	Lac Operator
TTTGTTTAACTTTAAGAAGGAGA	RBS

CATCATCATCATCAC	6x His (His-Tag)
CTGGTGCCGCGCGGCAG	Thrombin Site
CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTG	T7 Terminator

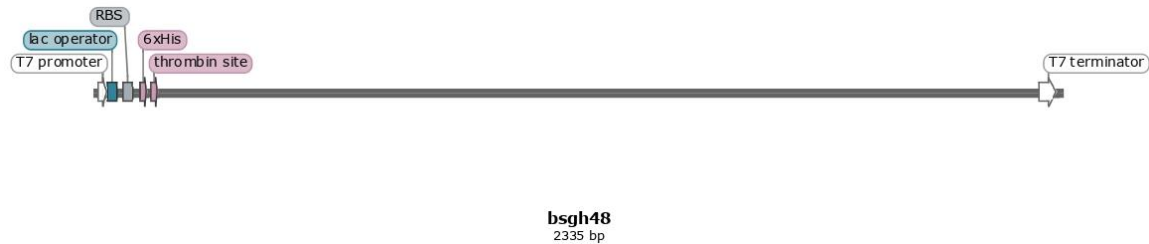


Figure 8. *bsgh48* depicted by the use of SnapGene.

The start codon was originally the “TTG” in the *B. safensis* strain. So there were two genome segments isolated, one with the original start codon and one with the “ATG” codon because it was not clear, at this point, which one would be expressed in the *E. coli* BL21 strain.

The amino acid sequence that was coded from the above DNA sequence is the following:

MSNKERFLTLYHQIKSDANGYFSPEGIPYHSIETLICEAPDYGHMTTSEASYWLWLEVLYGHYTGDWSKLEV
 AWDNMEKYIIPVNEDGHDEQPHMNAYNPSSPATYAAEKFPDQYPSQLTGARSAGQDPIDSELKSTYGSNE
 TYLMHWLLDVDNWWYGYGNLLNPSHKAAYVNTFQRGPQESVWESIPHSQDDKSFGKPNEGFMSLFTKENQ
 APAAQWRYTNATDADARAIQAMYWAKELGYNKPAYLDKAKKMGDFLRYGMYDKYFQTIGSGKQGNPYPG
 NGKGGCHYLMAWYTSWGGGLGEYANWSWRIGXSHCHQGYQNPVAAYALSTDKGGLKPSSATGASDWEK
 TLKRQLEFYVWLQSKEGAIAGGATNSWNGDYSAYPAGRSTFYDMAYEDAPVYLDPPSNNWFGMQAWPM
 ERVAELYIFVKDGDKTSENVQMAKSAITKWVNYALDYIFIGSRPVSDEEGYFLDDQGQRILGGTNVSVATTSA
 PGEFWLPGNIAWSGQPDWTWNGFQSATGNPNLTAVTKDPTQDTGVLGSLAKALIFFAAATKLETGDYTALGA
 RAKDAAAQLLEVAWNYNDGVGIVTEEDREDYDRFFRKEVYFPNGWNGTFTGQGNQIPGASTIPSDPQRGGN
 GVYTSFADLRPKIKQDPAWSSLESKYQSSFNEATGKWENGAPVFTYHFRWSQVDMATAYAEYHRLIN*

This amino acid sequence was entered in the ProtParam ExPASy tool (<https://web.expasy.org/protparam/>) to collect some general information about it, which is mentioned in Table 5.

Table 5. ProtParam ExPASy tool results for BsGH48 amino acid sequence.

ProtParam ExPASy tool results for BsGH48's amino acid sequence	
Number of amino acids	701
Molecular weight	78576.89
Theoretical pI	4.99
Total number of negatively charged residues (Asp + Glu)	82

Total number of positively charged residues (Arg + Lys)	56
Estimated half-life	>10 hours (<i>Escherichia coli</i> , in vivo)
Instability index (II)	35.43 (this classifies the protein as stable)
Aliphatic index	56.62
Grand average of hydropathicity (GRAVY)	-0.644

Then, the DNA sequence of *bsgh48* was entered in UniProt BLAST (<https://www.uniprot.org/blast>), an online tool that enables the comparison of a new sequence with the already existing ones in the UniProt library. The amino acid that had not been identified and was mentioned as 'X' in the sequence above, was replaced with an alanine. Alanine is a nonpolar amino acid and is the amino acid found, on this point, in the other similar proteins to BsGH48. The comparison of these sequences revealed that *bsgh48* indicates 98% homology with the sequence of an exoglucanase-2 from *Bacillus safensis* (Satomi et al. 2006) and 72.1% with the sequence of a protein of Glycoside Hydrolase Family 48 from *Bacillus licheniformis* (Veith et al. 2004; Rey et al. 2004). The amino acid sequences from the proteins are listed below:

- BsGH48 *Bacillus safensis* (ATHUBA63)
- Exoglucanase-2 from *Bacillus safensis* (ATCC BAA-1126): An exoglucanase-2 (Exoglucanase-2_*Bacillus safensis*) that is predicted (Satomi et al. 2006).
- GH Family 48 from *Bacillus licheniformis* (ATCC 14580/DSM 13): A protein sequence of a processive cellulase that regards evidence at the protein level regarding the UniProt library (Evandro Ares de Araújo et al. 2018; Evandro A. Araújo et al. 2021).
- BpCel48B *Bacillus pumilus* (SAFR-032): A crystallized cellulase (You et al. 2023).
- BpGH48 *Bacillus pumilus* (SH-B9): A biochemically characterized cellulase (Ogonda et al. 2021).

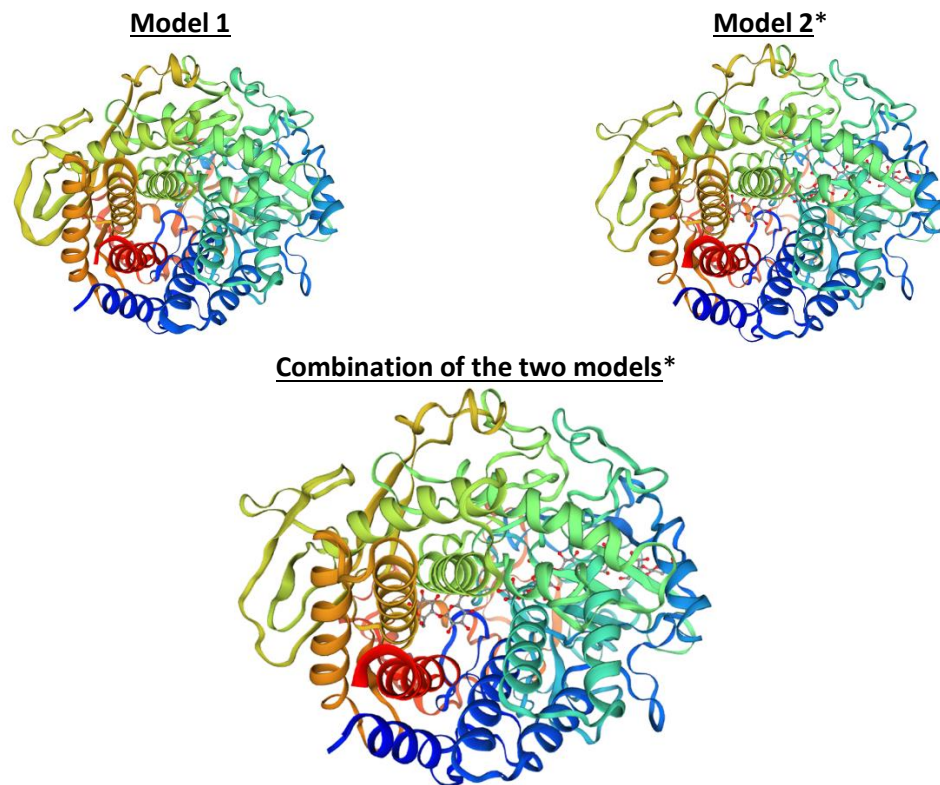
were aligned using the Clustal Omega. The result of the alignment is depicted in the following Figure 9. Taking into consideration that the amino acid sequence of BsGH48 was the reference sequence, the exoglucanase-2 sequence indicated a 98.0% percent identity, the sequence of *B. licheniformis* indicated a 73.2%, the BpCel48B sequence a 95.0% and the BpGH48 a 95.3%. All the sequences indicated a 97.1% percent coverage. Generally, the difference between the reference sequence and the first two is not significant.

GH_Family_48_Bacillus_licheniformis_ATCC_14580/DSM_13
BsGH48_Bacillus_safensis_ATHUBA63
Exoglucanase-2_Bacillus_safensis_ATCC_BAA-1126
BpCel48B_Bacillus_pumilus_SAFR-032
BpGH48_Bacillus_pumilus_SH-89

LIGNA	704
LIN--	722
LIN--	701
lin--	701
lin--	701

35

The next step was the visualization of the BsGH48 protein sequence using the Swiss Model Tool ExPASy (<https://swissmodel.expasy.org/interactive>). Although, the amino acid sequence of BsGH48 contains an 'X', so to construct the tertiary structure of the protein it was again substituted by an 'A'. Two models were created, the first one was based on the template of a Glycoside hydrolase (A0A267XE65.1.A) which is an AlphaFold DB model of A0A267XE65_9BACI, a gene from the *Bacillus sp.* 7788. The second one was based on the template of the Structure of *Bacillus pumilus* GH48 which structure was discovered by the method of X-RAY DIFFRACTION 2.00 Å (Brunecky et al. 2017b). The first model indicated 96.15% sequence identity, 0.62 sequence similarity, and 0.97 coverage. The second model indicated 95.01% sequence identity, 0.62 sequence similarity, and 0.97 coverage. The Swiss Model Tool ExPASy provides the opportunity to combine the two models. The tertiary structures of BsGH48 are depicted in the following Figure 10.



*the structures in complex with cellobiose and cellohexaose.

Figure 10. The tertiary structures of BsGH48.

All the GH48 protein structures exhibit a similar overall backbone. They all present a typical (a/a)₆ barrel which consists of six mutually parallel α -helices, forming an inner core, and six peripheral α -helices, forming an outer shell. Additionally, they share long loops connecting the

N-terminus of each inner helix. The active site topology, that forms a complex with the oligosaccharides, consists of two parts. A tunnel-like substrate binding part and an open-cleft product binding part. These similarities are present in the 3D structure of BsGH48 generated from the combination of the two models, as depicted in Figure 11.

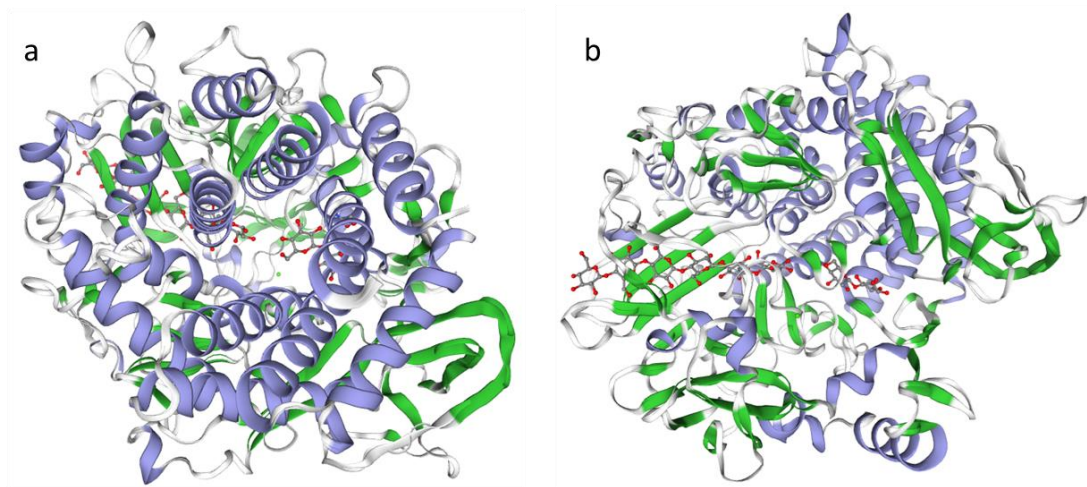


Figure 11. The 3D structure of BsGH48 was generated from the combination of the two models using the Swiss Model Tool ExPASy. The 2^o structure is highlighted in different colours. In Figure a. the (a/a)6 barrel is visible and in Figure b. the active site binds with the substrate cellohexaose and with the product, cellobiose is also visible.

The GH48 proteins from *Bacillus* sp. usually present longer loops compared to the other GH48 proteins. Both BpCel48B of *B. pumilus* and BICel48B of *B. licheniformis* seem to verify this. BpCel48B has eight longer loops and BICel48B has three (Brunecky et al. 2017b; Evandro A. Araújo et al. 2021). The loops near the exit of the active site do not affect the product inhibition. However, they might be responsible for the lower thermostability of these proteins. Regarding the active site, most of the residues consisting of the tunnel-like part are aromatic residues. Of these 36 residues, 27 are universally conserved and the others are highly conserved (You et al. 2023; Brunecky et al. 2017a). The active site's conservation is depicted in Table 14. The GH48 enzymes in substrate recognition and correct folding have three types of amino acids participating: hydrophobic stacking interactions, hydrogen bonding, and calcium coordination residues (Sukharnikov et al. 2012).

2. Genetic modification of BL21 (DE3) *Escherichia coli* for the overexpression of BsGH48

2.1. Isolation of the *bsgh48*

Both, the *bsgh48*(ATG) and the *bsgh48*(TTG) were isolated by a *B. safensis* single colony from an LB agar plate with colony PCR, using the primers from Table 1. The PCR products were analyzed by agarose gel electrophoresis and their length was evaluated. In Figure 12 the length of the DNA segments can be estimated to be a little bigger than 2000 bp. This value is also verified by the bioinformatics analysis according to which they were measured to be 23335 bp. These will be the “inserts” in the following genetic modification procedure.

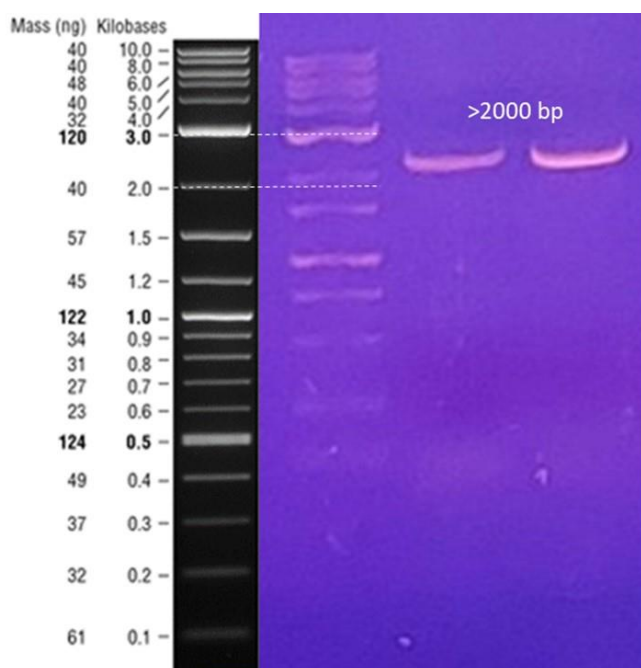


Figure 12. Electrophoresis of *bsgh48*(ATG) and *bsgh48*(TTG) respectively.

2.2. Genetic modification of BL21 (DE3) *E. coli*

The vector was isolated from an *E. coli* DH5a single colony from an LB agar plate by the same method as the insert and its length is known to be 5708 bp. The absorbance of both *pet15b16s* and *bsgh48* was measured at 260 and 280 nm, before the digestion, to estimate the proportions of the restriction enzymes and the DNA molecules and after to estimate the proportions for the ligation. The results are listed in Table 6.

Table 6. Purity and concentration of the inserts and the vector.

Before the digestion	Concentration ($\mu\text{g/mL}$)	Purity	
		260/280	260/230
<i>bsgh48</i> (TTG)	82.50	1.827	1.472
<i>bsgh48</i> (ATG)	70.87	1.774	1.152
<i>pet15b16s</i>	169.60	1.725	1.030
After the digestion			
<i>bsgh48</i> (TTG)	16.33	1.732	0.037
<i>bsgh48</i> (ATG)	21.73	1.501	1.359
<i>pet15b16s</i>	5.765	1.825	0.153

After the digestion of the vector and the insert with the restriction enzymes *NotI* and *SpeI*, the ligation procedure took place. The *E. coli* BL21 (DE3) was successfully transformed with the product of ligation as shown in Figure 13.

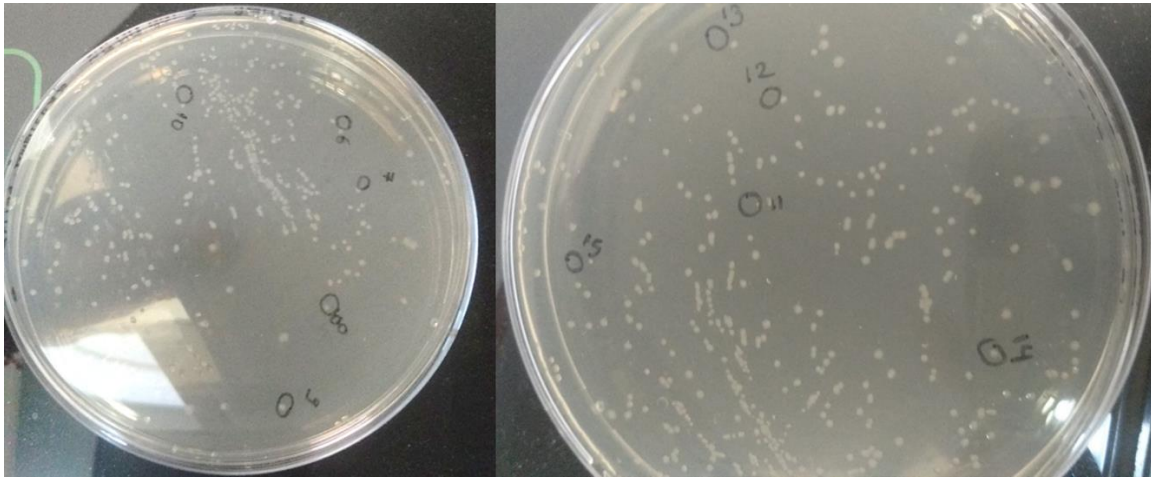


Figure 13. *E. coli* BL21 (DE3) transformed with *pet15b16s-bsgh48* plasmid vector. On the left is the LB agar plate culture containing the *pet15b16s-bsgh48*(TTG) vector and on the right is the culture with the *pet15b16s-bsgh48*(ATG) vector.

2.3. Overexpression of the BsGH48

For the overexpression of the BsGH48 two methods were tested, the “Overnight Batch” and the “Fast Batch” method. The following figure, [Figure 14](#), depicts the SDS-Page electrophoresis of the proteins isolated from each batch. The “Overnight Batch” method is more efficient than the other.

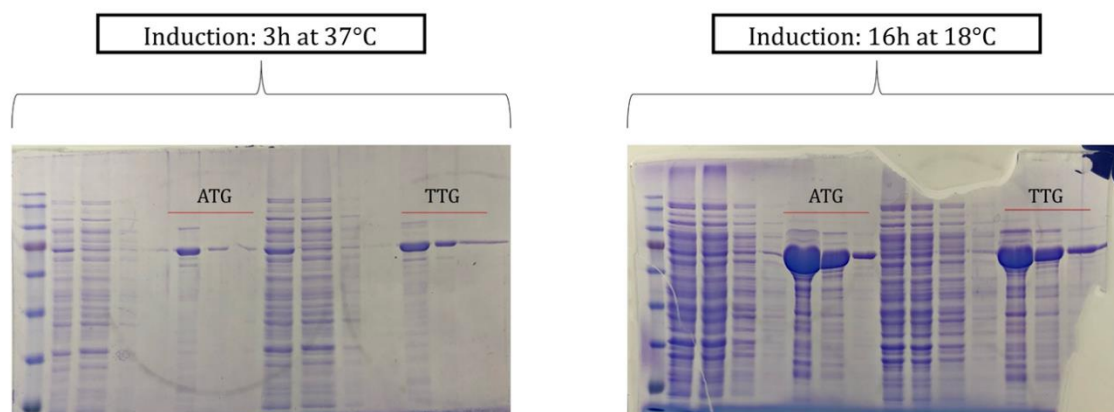


Figure 14. Two methods were examined for the overexpression of the BsGH48.

In Figure 14 the expression of the two different protein molecules can be easily detected. Both proteins are equally expressed from the *E. coli* BL21 (DE3) strain. Their enzymatic activity was tested with PASC and no great differences were noted (Table 7). So after the overexpression of these protein molecules, the different samples were examined as the same protein.

Table 7. The comparison between the two protein molecules of their catalytic activity.

	Protein concentration	Catalytic activity
TTG		
sample 1	14.73 mg/ml	0.1405 U/mL
sample 2	19.29 mg/ml	0.1732 U/mL
ATG		
sample 1	12.56 mg/ml	0.1058 U/mL
sample 2	18.22 mg/ml	0.1680 U/mL

3. Characterization of the BsGH48

3.1. Determination of BsGH48's molecular weight

An SDS-Page electrophoresis was conducted to determine the molecular weight of BsGH48 using a prestained protein ladder (MW 10-180 kDa). The molecular weight is estimated at 74 kDa as depicted in Figure 15.

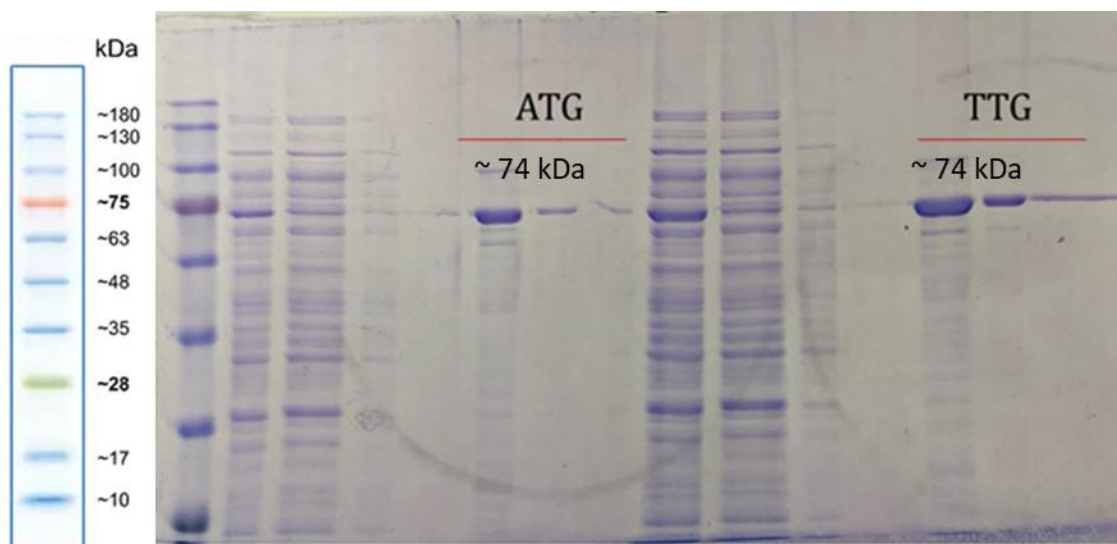


Figure 15. SDS-Page electrophoresis of the BsGH48 and a prestained ladder to determine its molecular weight.

3.2. Biochemical Characterization of BsGH48

3.2.1. Optimum pH

BsGH48's highest activity was observed at citrate phosphate buffer pH 5.0. However, at tris-HCl buffer pH 8.0 it reached 96.7% of the maximum relative activity, as depicted in Figure 16. The difference between these values is not significant. So BsGh48 technically presents two optimum pH values. The relative activity remains high, with 97.1% of the maximum activity at phosphate buffer pH 5.0. It gradually decreases at phosphate buffer presenting 84.7% at pH 6.0, 73.4% at pH 7.0, and 57.3% at pH 8.0 of the maximum activity. It is true that at pH 8.0 of a different buffer, the relative activity is a lot lower than at the same pH of the tris-HCl buffer. The relative activity at pH 9.0 is 61.7% of the maximum activity, similar to the 62.7% presented at pH 4.0. It is only dropped below 50% at pH 3.0 which presents a 3.8% of the maximum activity. The BsGh48 cannot be identified either as acidophilic nor as alkaliphilic, because at lower pH values than 5.0 and higher than 8.0, its relative activity dropped significantly. Bibliographically there are references for enzymes with two optimum pH values, like a bovine milk acid phosphatase which indicates an optimum pH range between 4.5 and 6.0 and an additional pH optimum around 3.0 for different buffers (André et al. 2017). However, there is no reference for a cellulase having two optimum pH, one in acidic and one in alkaline pH range. The BpGH48 also exhibits optimal activity at a pH of 5.0 and low activity at higher pH values than 8.0, even if *B. pumilus* was isolated from an alkaliphilic environment (Ogonda et al. 2021). BICel48B shows the same activity profile as BpGH48, with an optimum pH of 5.0 (Evandro Ares de Araújo et al. 2018).

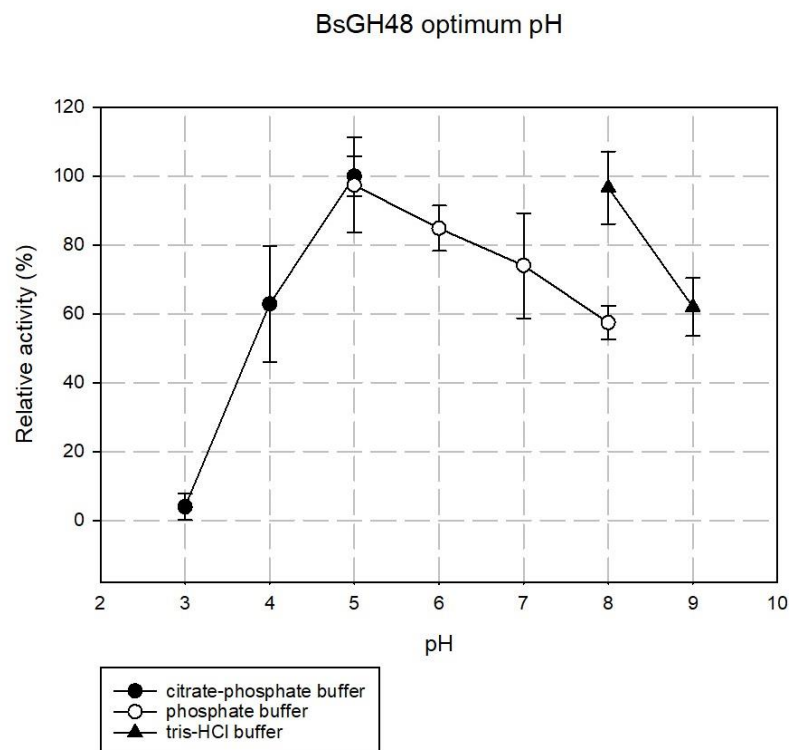


Figure 16. The optimum pH of BsGH48.

3.2.2. Optimum temperature

The optimum temperature of action for BsGh48 is 50 °C, as depicted in Figure 17. The relative activity of BsGH48 remains high at 91.5% of the maximum activity at 40 °C. It is steadily decreasing at the lower temperatures, indicating 42.8% and 18.6% of the maximum activity at 30 °C and 20 °C respectively. However, at temperatures higher than 50 °C the decrease is significant. The relative activity at 60 °C is estimated at 54.2% of the maximum and at temperatures 70-80 is lower than 10%. BsGh48 shows more than 50% of the maximum activity only at 30-50 °C temperature range. Similar behavior is demonstrated by BICel48B of *B. licheniformis* which presents the highest relative activity at 60 °C, but it only indicates >50% of the maximum one at the short range of 50-60 °C. At temperatures higher than 60 °C its relative activity decreases lower than 40% of the maximum activity (Evandro Ares de Araújo et al. 2018).

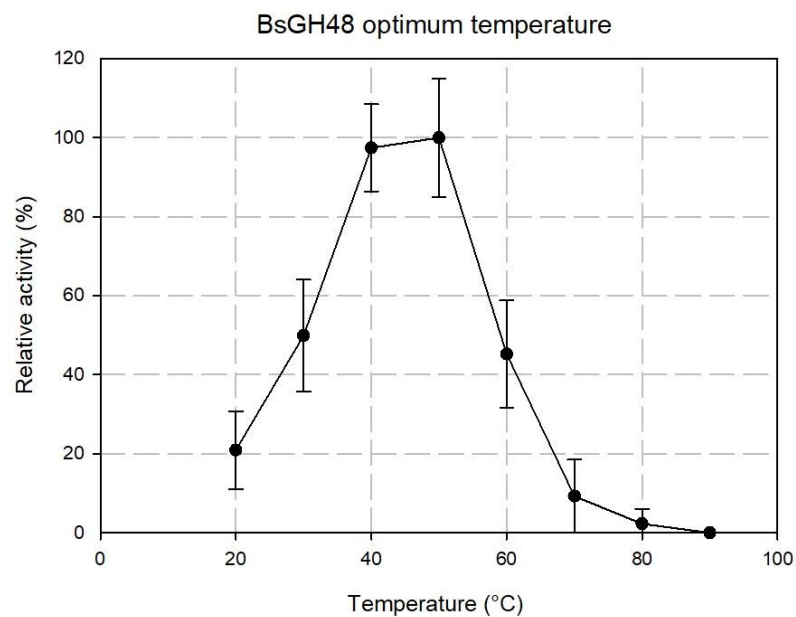


Figure 17. The optimum temperature of BsGH48.

Generally, the optimal activity of GH48 enzymes was mainly registered within temperatures between 50 and 70 °C, as presented in Table 8, but there are exceptions, too. The Cel48C and Cel48 enzymes present an optimum temperature at 45 °C and CbCelA presents its highest relative activity at 85 °C.

Table 8. The biochemically characterized enzymes of the GH48 family.

Microorganisms (ID)	Protein	MW (kDa)	Optimum		Km	Stability			Citations
			T _{opt}	pH _{opt}		T _M	pH	t _{1/2}	
<i>Bacillus safensis</i> (ATHUBA63)	BsGH48	~74	50 °C	5.0					Present study
<i>Bacillus licheniformis</i> (ATCC 14580/DSM 13)	BlCel48/ BlCel48B	77.2	60 °C	5.0	Nr	73 °C	retained about 65 to 90% of the maximum activity at pH in the range of 4.0-6.0	Nr	(Evandro Ares de Araújo et al. 2018)(Evandro A. Araújo et al. 2021)
<i>Bacillus pumilus</i> (SH-B9)	BpGH48	79.59	55 °C	5.0	Nr	Nr	low activity at high pH	Nr	(Ogonda et al. 2021)
<i>Bacillus pumilus</i> (SAFR-032)	BpumGH48 / BpCelGH48	Nr	37 °C	5.5	Nr	45 °C	Nr	Nr	(Brunecky et al. 2017b)
<i>Paenibacillus</i> sp. (BP-23)	Cel48C	118	45 °C	6.0	Nr	Nr	Nr	Nr	(Sánchez, Javier Pastor, and Diaz 2003)
<i>Myxobacter</i> sp. (AL-1)	Cel48	81	45 °C	6.0	Nr	is rapidly denatured above 60 °C	Nr	Nr	(Ramírez-Ramírez et al. 2008)(Evandro Ares de Araújo et al. 2018)
<i>Clostridium thermocellum</i>	CtCelS	82	70 °C	5.5	Nr	Nr	Nr	Nr	(Kruus et al. 1995)
<i>Clostridium thermocellum</i>	CtCel48Y	Nr	65 °C	7.0	Nr	65 ^a °C 50% or more activity between 50 and 71°C and none above 75°C	Nr	Nr	(Berger et al. 2007)
<i>Caldicellulosiruptor bescii</i>	CbCelA	Nr	85 °C	Nr	Nr	80 °C retained high activity at all temperatures tested,	Nr	Nr	(Brunecky et al. 2013)

						converting 60% of glucan at 85°C			
<i>Thermobifida fusca</i>	TfCel48A	104	50 °C	6.0- 6.5	Nr	65 °C activity was the same at 40 °C and 50 °C and declined to <33% at 60 °C	retained >50% of their activity from pH 5.0 to pH 9.0	Nr	(Irwin, Zhang, and Wilson 2000)
<i>Clostridium cellulolyticum</i>	CcCelF (endonucle ase)	79	55°C	5.5- 6.0	2.4 g/li ter	is rapidly denatured at 50 °C	Nr	30 min	(Reverbel- Leroy et al. 1997)
<i>Clostridium cellulovorans</i>	CcExgS	77.7	Nr	Nr	Nr	Nr	Nr	Nr	(C.-C. Liu and Doi 1998)
<i>Clostridium stercorarium</i>	CsCel148Y/ Avicelase 11	87	70 °C	5.0- 6.0	Nr	highly thermostable	retained 85% of the maximal activity at pH 7.0	Nr	(BRONNENMEI ER, RÜCKNAGEL, and STAUDENBAU ER 1991)
<i>Anaerocellum thermophilum</i>	CelA (bifunctional and heavily glycosylated)	et al.	85-95 °C	5.0- 5.5	Nr	Nr	Nr	40 min	(Zverlov et al. 1998)
Nr: no reference									

3.2.3. Substrate specificity

The substrate specificity of BsGH48 was examined using 16 different substrates. Specifically, the substrates studied were PASC, microcrystalline cellulose (Avicel), carboxymethyl cellulose (CMC), filter paper, β -Glucan, 2NP- β -D-Galactopyranoside, pNP- α -D-Galactopyranoside, pNP- α -D-Glucopyranoside, 4NP- β -D-Xylopyranoside, lichenan, arabinoxylan, xylan-oat spell, xylan birchwood, xylan beechwood, and a-cellulose. The relative activity of BsGH48 was considered to be the one with PASC as substrate, as mentioned in Table 9. However, the relative activity with Avicel was more than twice as high as with PASC. The next highest relative activity was 17.5% of the maximum one and it was presented with lichenan. A 16.5% and 14.5% of the maximum relative activity was indicated with filter paper and arabinoxylan respectively. A low relative activity was presented with all the xylans. Although, with nitrophenyl-pyranoside substrates, there was a null activity only with two of them. A null relative activity was presented with both CMC and a-cellulose, too. BsGH48 did not react with β -Glucan. The enzyme presented a really low relative activity with hemicellular and nitrophenyl-pyranoside substrates. However, from the highly structural-stable substrates BsGH48 only reacted with Avicel, presenting a twice bigger relative activity than the maximum one. BsGH48 appears to be a β -1,4-exoglucanase with great specificity.

Table 9. The substrate specificity of BsGH48.

Substrate	Relative activity (%)
PASC	100.0 $\pm$ 14.4
Avicel	226.6 $\pm$ 33.4
CMC	8.5 $\pm$ 1.9
Filter paper	16.9 $\pm$ 16.4
β -Glucan	ND
2NP- β -D-Galactopyranoside	5.2 $\pm$ 2.0.6
pNP- α -D-Galactopyranoside	ND
pNP- α -D-Glucopyranoside	5.0 $\pm$ 0.4
pNP- β -D-Glucopyranoside	ND
4NP- β -D-Xylopyranoside	ND
Lichenan	17.5 $\pm$ 5.53
Arabinoxylan	14.5 $\pm$ 9.8
XYLAN-Oat spell	6.5 $\pm$ 2.8
Xylan Birchwood	11.2 $\pm$ 0.2
Xylan Beechwood	11.5 $\pm$ 1.2
A-cellulose	7.5 $\pm$ 7.2
ND: no activity detected	

3.2.4. Effect of various modulators

The effect of some metal compounds and two organic compounds, EDTA and SDS, on BsGH48 is depicted in Figure 18. The enzyme was incubated with these modulators for 30 min and 1 hour. BsGH48 presents more than the triple relative action in the presence of manganese ions (Mn^{2+}). After a 30-minute incubation in a MnSO_4 buffer, its relative activity was 354.1% of the one in the absence of any modulator, which remained approximately the same after a 1-hour incubation. Generally, the relative activity of BsGH48 was enhanced by the majority of modulators. In the presence of CuCl_2 its relative activity was 147.8% of the standard one after a 30-minute incubation and it reached 223.4% after a 1-hour one. Accordingly, the relative activity of BsGH48 not only increased in the presence of MnCl_2 but almost doubled after 1-hour incubation. It seems that Mn^{2+} and Cu^{2+} divalent ions enhance significantly the activity of BsGH48.

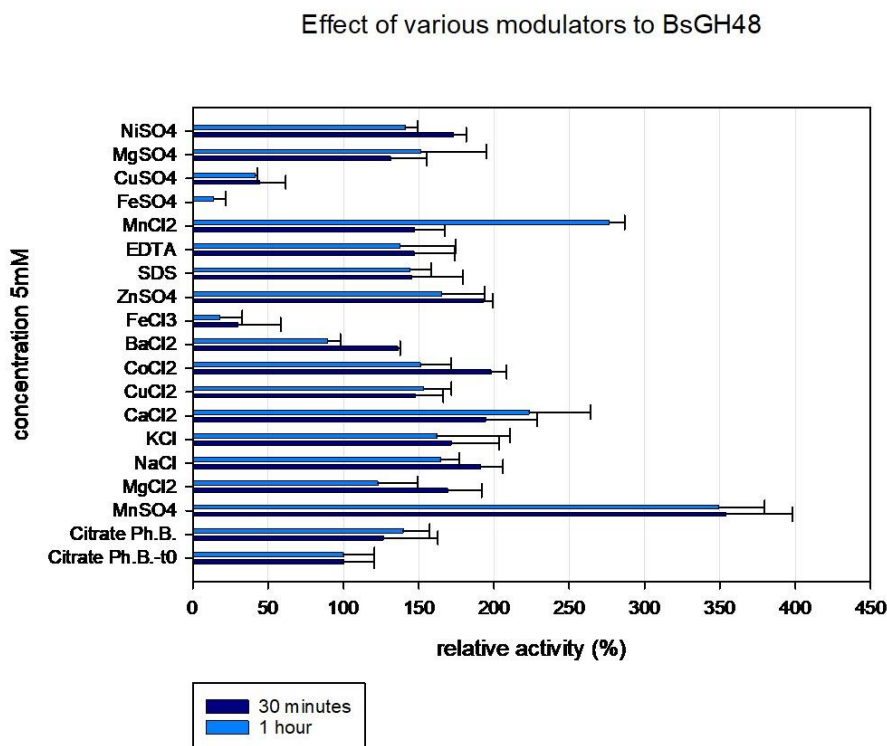


Figure 18. Effect of various modulators on BsGH48.

Some modulators decrease the relative activity of BsGH48. Ferrous ions, Fe^{2+} and Fe^{3+} , reduce it more than any modulator, with a zero measurement after a 30-minute incubation in the presence of FeSO_4 . The low relative activity percentage observed with CuSO_4 remained steady after a 30-minute and a 1-hour incubation. However, the Cu^{2+} ions enhanced the relative activity

of BsGH48 when they participated in CuCl_2 , making the ion SO_4^{2-} probably the responsible one for this decrease. BsGH48's relative activity was increased in the presence of BaCl_2 after a 30-minute incubation but it decreased one and a half times after a 1-hour incubation.

Even if ions like Mn^{2+} enhance the relative activity of BsGH48 more than three times, BsGH48 does not require metal ions as activators. EDTA inhibits some enzyme activities that require metal ions as activators and BsGH48's activity was not reduced in its presence (Mónico et al. 2017). This enzyme indicated a great resilience in the presence of SDS. This organic compound causes an enzyme to lose its higher-order structures and become linear. Since it is anionic, it binds to all the positive charges on a protein, effectively coating the negative charged proteins (Kaspersen et al. 2017). Examining the 3D structure of BsGH48 and its amino acid sequence, it is shown that the negatively charged amino acids are more than the positive ones (Lys:36; Arg:22; His:20; Glu:39; Asp:43), as depicted in Figure 19. This might explain BsGH48's resilience to the SDS buffer.

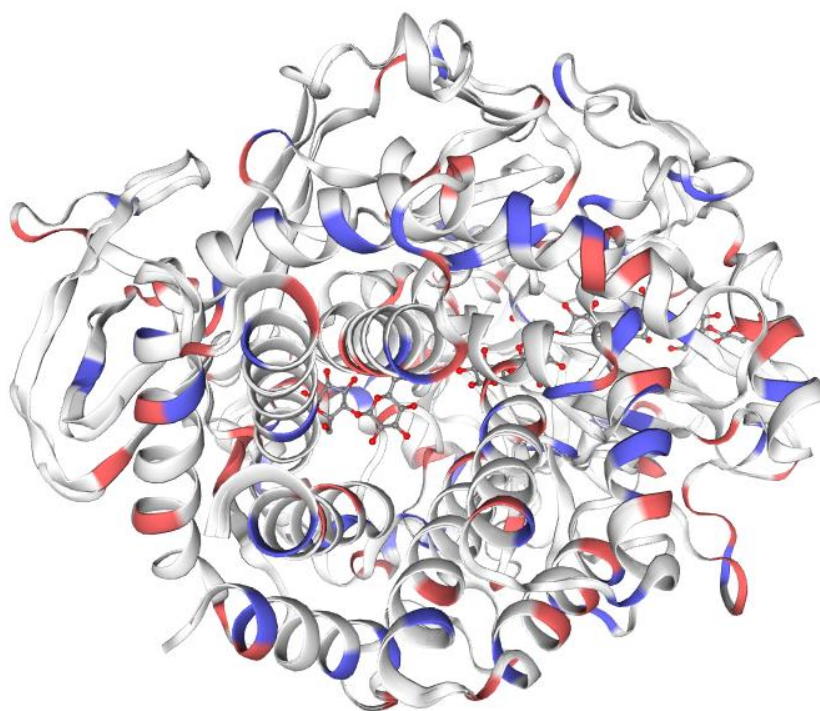


Figure 19. The depiction of BsGH48 charged. The red colour is for the negative charged amino acids and the blue for the positive ones.

BICel48B's reposted structure indicates that calcium ions bond to the tunnel-like part of the active site. These ions are involved in binding and stabilizing the two loops and the helix-turn

which are found at the catalytic exit. It seems that the flexibility of those loops occurs to these ions, regarding the fact that in the presence of 5mM CaCl_2 , the activity of BICel48B is enhanced by 29% (Evandro A. Araújo et al. 2021).

3.2.5. Kinetic constants

The kinetic constants of BsGH48 were calculated separately based on experimental data derived from the hydrolysis of Avicel and PASC. These constants are K_m and V_{max} . These calculations were performed based on the double inverse Lineweaver-Burk plot which quantifies more accurately the Michaelis-Menten equation of enzyme kinetics. The Lineweaver-Burk plots for each substrate tested are depicted in Figure 20.

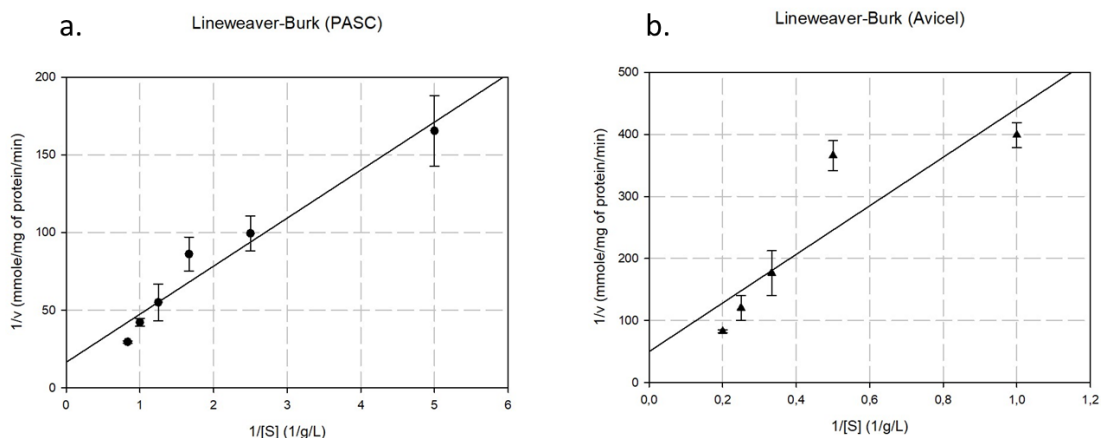


Figure 20. The Lineweaver-Burk plots for BsGH48. The substrate used for the plot (a) was PASC, and for the plot (b) was Avicel.

The kinetic constants derived from the Lineweaver-Burk plot are mentioned in Table 10.

Table 10. The kinetic constants of BsGH48 for Avicel and PASC.

Substrate	K_m (g/L)	V_{max} (mmole/mg of protein/min)
Avicel	9.552	0.025
PASC	1.867	0.061

The K_m value for the degradation of PASC is lower than the one for Avicel. This means that BsGH48 presents a greater relation with PASC as a substrate than with Avicel. There are not a lot of bacterial cellobiohydrolases the kinetics constants of whom were studied. Cel48A from *Thermobifida fusca* is the only cellulase of the 48 family that was studied kinetically. It presents a K_m value of 1.8 ± 0.3 mg/ml with Bacterial cellulose (BC) as the substrate (Kostylev 2013). Most of the enzymes whose kinetics were studied originate from fungi. For example, Cel7A from

mesophilic *Hypocrea jecorina* and thermophilic *Rasamsonia emersonii* present a K_m value of 122 ± 8 g/L and 76 ± 5.1 g/L respectively at 50 °C (Sørensen et al. 2015).

3.3. Processivity assay

The standard assay was conducted to measure the reducing sugars in the supernatant and the sediment of the reaction mixture. Using a glucose standard curve, the amount of the reducing sugars in the supernatant was 0,0660 mg/mL, and in the sediment was 0,0003 mg/mL. So it is clear that BsGH48 is not a processive enzyme. This conclusion was expected since exo-cellulases produce more than 93% of the total reducing sugar in the solution while endo-cellulases produce 30–40% of the total reducing ends in the insoluble substrate (Wilson et al. 2012). However, BICel48B cellulase from *B. licheniformis* is a processive cellulase of the GH48 protein family (Evandro A. Araújo et al. 2021).

3.4. Stability of BsGH48

3.4.1. Effects of Temperature

The thermal stability of BsGH48 was tested at the following temperatures: 40, 50, 60, 65, and 70 °C. Enzyme heat inactivation follows first-order kinetics as indicated in the equation below:

$$\ln \frac{A_t}{A_0} = -k_d \cdot t \quad (1)$$

where A_t (Units/mg of the protein) is the enzymatic activity of BsGH48 for the time t , A_0 (Units/mg of the protein) is the original enzymatic activity of BsGH48, t (min) is the incubation time, and k_d (min^{-1}) is the constant of thermal denaturation of BsGH48.

The thermal deactivation constants are calculated by plotting the enzyme residual activity measured in each temperature (A) versus the elapsed time and calculating the slope of each linear regression (Figure 21). Three constants were calculated, the constant of thermal denaturation of BsGH48 (k_d), the half-life ($t_{1/2}$), and the decimal reduction time or D-value (D).

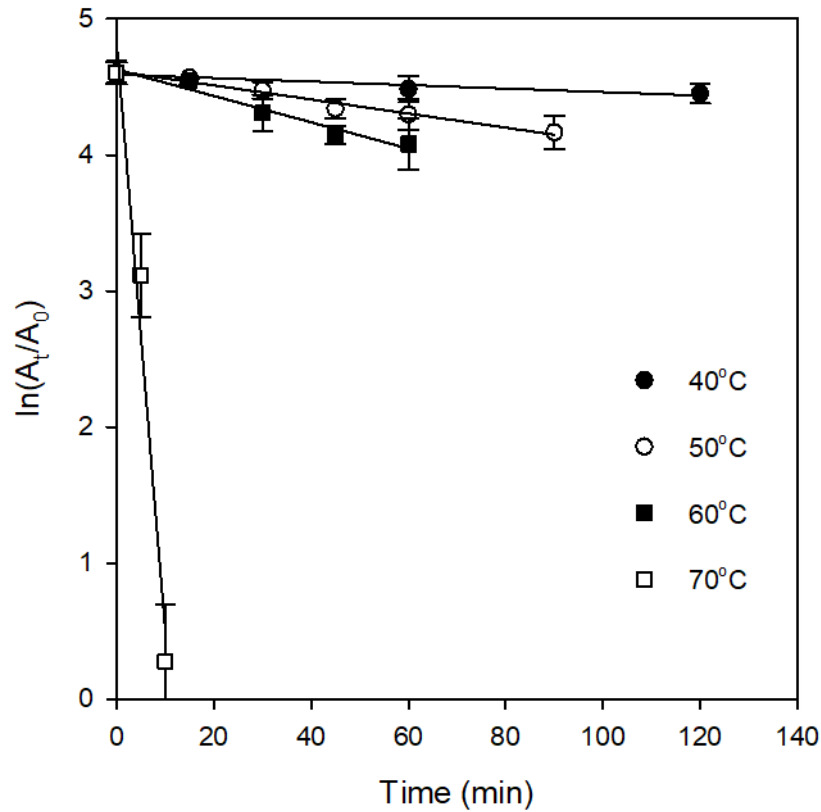


Figure 21. The thermal stability of BsGH48 at the temperature range of 40-70 °C.

The half-life is the time duration for an enzyme's activity at a certain temperature to be reduced at 50% and it is calculated based on the following equation:

$$t_{1/2} = \frac{0.6931}{k_d} \quad (2)$$

where $t_{1/2}$ (min) is the half-life of BsGH48, and k_d (min^{-1}) is the constant of thermal denaturation of BsGH48.

The D-value is the necessary time duration for an enzyme's activity at a certain temperature to be reduced at 90% regarding its primary value and it is calculated by equation number 3:

$$D = \frac{2.303}{k_d} \quad (3)$$

where D (min) is the decimal reduction time and k_d (min^{-1}) is the constant of thermal denaturation of BsGH48.

The thermal deactivation constants derived from the experimental data of BsGH48 are mentioned in Table 11.

Table 11. The thermal deactivation constants of BsGH48.

Temperature (°C)	k_d (min ⁻¹)	$t_{1/2}$ (min)	D (min)
40	1,3E-03	533.15	1771.54
50	5,2E-03	133.29	442.88
60	9,7E-03	71.45	237.42
65	2,6E-01	3.08	10.22
70	4,3E-01	2.00	5.00

The thermal inactivation of BsGH48 presents a steady increase in the first three temperatures tested: 40, 50, and 60 °C. However, in the final temperature, 70 °C, the enzyme is inactivated abruptly. These observations can be confirmed by the thermal deactivation constants which change size order at the last temperature tested. Specifically, BsGH48 displays a slow loss of enzymatic activity at 40 °C, indicating a half-life of 533.15 minutes, a D-valued of 1771.54 minutes, and a k_d of 0.0013 min⁻¹. The decline of enzyme activity at 50 and 60 °C was gradual, while the k_d value displays an increase of ~0.0040 at each temperature. The other two constants presented a similar behavior for these temperatures. At 70 °C, the quickest inactivation of the enzyme was observed as its activity was reduced to zero within the first 10 minutes of incubation. The half-life of BsGH48 at this temperature was 2.00 minutes, the D- value 5.00, and the k_d 0.4300 min⁻¹.

BlCel48 of *Bacillus licheniphormis* was significantly stable at the temperature range of 40-50 °C. it preserved 80% of its enzymatic activity for 48 hours and 12 hours at 40 °C and 50 °C, respectively. However, BlCel48's enzymatic activity at 60 °C was reduced to less than 40% after a 1-hour incubation (Evandro Ares de Araújo et al. 2018). BlCel48 displayed a similar behavior to BsGH48. On the other hand, both Cel48A and Cel48AcD presented similar activities at 40 °C and 50 °C overnight assays, but their activities declined to ~30% at 60 °C (Irwin, Zhang, and Wilson 2000).

The thermal inactivation of the enzyme process is a two-step procedure. In the first step, the enzyme is converted to an unstable intermediate transition state, INT[#], which is in equilibrium with the native enzyme (N) at a given temperature and, therefore, can fold back to its original state upon cooling. As the temperature increases, the enzyme undergoes some permanent changes, for example, aggregation due to intermolecular hydrophobic interactions upon unfolding, chemical degradation, subunit dissociation, or disulfide-bridges exchange. Thus, a certain amount of the enzyme will leave the $N \rightleftharpoons INT^{\#}$ equilibrium and will be denatured in an irreversible stage. The enzyme denaturation procedure is presented as $N \rightleftharpoons INT^{\#} \rightarrow D$. (Saqib and

Siddiqui 2018). The stability of a protein molecule can be characterized as the equilibrium between stabilizing and destabilizing formations within its chemical structure. For example, hydrophobic interactions within the three-dimensional folding can reduce its stability while other interactions such as disulfide or hydrogen bonds increase its rigidity (Ortega et al. 2004). The energy required for the enzyme to attain the denatured form is the thermal inactivation energy ($E_{(a)d}$). The higher the value of the $E_{(a)d}$ is, the more thermostable the enzyme is. The $E_{(a)d}$ of BsGH48 was calculated by the Arrhenius plot, **Error! Reference source not found.**, and its value was $159.7 \pm 7,3$ kJ/mole. This plot was derived from the Arrhenius equation (4):

$$\ln k = -\frac{E_a}{R} \left(\frac{1}{T} \right) + \ln A \quad (4)$$

Arrhenius

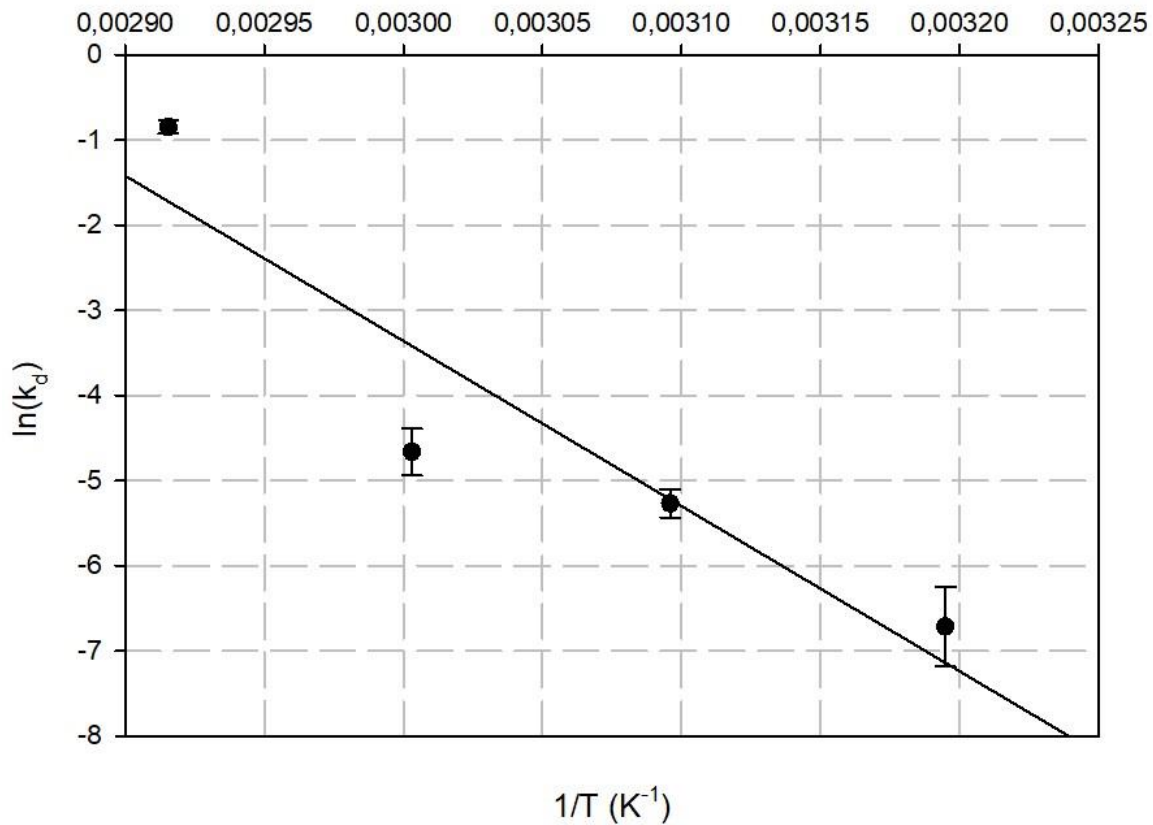


Figure 22. The Arrhenius plot for the calculation of BsGH48's $E_{(a)d}$.

Based on the thermal inactivation energy ($E_{(a)d}$) the thermodynamic parameters of thermal deactivation of BsGH48 can be calculated. These calculations are derived following the equations below (Eyring & Stearn):

k_d (sec^{-1}) is thermal deactivation constant, which is calculated by equation 5.

$$k_d = \left(\frac{k \cdot T}{h} \right) \cdot e^{(-\Delta H^*/R \cdot T)} \cdot e^{(\Delta S^*/R)} \quad (5)$$

where T (K) is the absolute temperature, R ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) is the global gas constant, h ($1.104 \times 10^{-35} \text{ J} \cdot \text{min}$) is the Plank constant, and k ($1.38 \times 10^{-23} \text{ J} \cdot \text{K}^{-1}$) is the Boltzmann constant.

ΔH^* ($\text{kJ} \cdot \text{mol}^{-1}$) is the deactivation enthalpy change, which is calculated by equation 6.

$$\Delta H^* = E_{(a)d} - R \cdot T \quad (6)$$

where T (K) is the absolute temperature, $E_{(a)d}$ is the thermal inactivation energy, and R ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) is the global gas constant.

ΔS^* ($\text{kJ} \cdot \text{mol}^{-1}$) is the activation entropy change, which is calculated by equation 7.

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \quad (7)$$

where T (K) is the absolute temperature, ΔH^* ($\text{kJ} \cdot \text{mol}^{-1}$) is the deactivation enthalpy change, and ΔG^* ($\text{kJ} \cdot \text{mol}^{-1}$) is the Gibbs free energy of deactivation, which is calculated by equation 8.

$$\Delta G^* = -R \cdot T \cdot \ln \left(\frac{k_d \cdot h}{k \cdot T} \right) \quad (8)$$

where R ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) is the global gas constant, T (K) is the absolute temperature, k_d (sec^{-1}) is thermal deactivation constant, h ($1.104 \times 10^{-35} \text{ J} \cdot \text{min}$) is the Plank constant, and k ($1.38 \times 10^{-23} \text{ J} \cdot \text{K}^{-1}$) is the Boltzmann constant.

The deactivation enthalpy change (ΔH^*) is the measure of the degree and/or intensity of the thermal disruption of the noncovalent linkages in enzymes, forming a transition state of the enzyme inactivation ($\text{INT}^\#$). Thus, the higher its value is, the larger will be the number of noncovalent bonds that need to break for the enzyme to lose its structure. A large number of noncovalent bonds directly translates to greater enzyme stability. The Gibbs free energy of deactivation (ΔG^*) is the energy required for the enzyme to enter the denaturation phase. It includes the other two thermodynamic parameters, so it is a great aggregate index of the thermal stability of an enzyme. The higher its value is, the higher will be the enzyme stability. The activation entropy change (ΔS^*) is directly related to ΔH^* . It can indicate the degrees of

solvation and compactness in the protein molecular structure, and the enzyme and solvent disorder due to thermal increase (Gouzi, Depagne, and Coradin 2012; Ibrahim et al. 2021).

Table 12. The thermodynamic parameters of thermal deactivation of BsGH48.

Temperature (°C)	ΔH^* (kJ/mole)	ΔG^* (kJ/mole)	ΔS^* (J/mole·K)
40	157.1	104.7	167.3
50	157.0	104.4	162.8
60	156.9	106.0	152.9
70	156.9	98.5	170.2

The change in deactivation enthalpy during deactivation of BsGH48 is measured between 156.9 kJ/mole and 157.1 kJ/mole in the range of temperatures tested, as indicated in Table 12. Thermal inactivation can be directly correlated with the breakage of noncovalent bonds within the three-dimensional folding of the molecule that causes it to unfold. The change in enthalpy during deactivation is a direct measure of the breaking of such bonds as it has been calculated that the breakage of a methyl group ($-\text{CH}_3$) from a larger structure requires approximately 5.4 $\text{kJ}\cdot\text{mole}^{-1}$ (Pace 1992). Therefore, based on the ΔH^* values it can be calculated that on average 29 noncovalent bonds break during the denaturation process of BsGH48. The ΔH^* presents to be independent of temperature, therefore, there is no change in enzyme heat capacity. The change in free Gibbs energy (ΔG^*) during deactivation is the most direct marker of an enzyme's thermal stability. Low values of ΔG^* translate to spontaneous unfolding of the protein structure while high values translate to the opposite. The value of ΔG^* can be considered steady at the temperature range 40-60 °C. The values of ΔG^* display a drop by 70 °C during the increase of test temperature as expected, as the temperature rise directly results in easier protein denaturation. The change in entropy during deactivation (ΔS^*) can be directly correlated with the change in enthalpy and by extension with the stability of an enzyme. It functions as the opposite measure to the change in enthalpy, representing the natural tendency of the molecule to unfold. A combination of low change in entropy values and high change in enthalpy values denotes a highly stable enzyme. Moreover, positive change in entropy is a marker that during the denaturation process, the enzyme does not present aggregates of various sizes (Ortega et al. 2004). The ΔS^* value was measured between 162.8 J/mole-K and 170.2 J/mole-K. The temperature where ΔS^* has the lowest value, and high ΔH^* and ΔG^* values is 60 °C. This is the temperature where BsGH48 is more thermostable. Although, its thermostability at 60 °C does not differ a lot at 40 °C and 50 °C.

3.4.2. Effects of pH

BsGH48 presents great stability at pH ranging from 5.0 to 9.0. The enzyme retains more than 80% of its relative activity indicated from the standard assay, after a 24-hour at each pH point tested. BsGH48's highest stability is shown at pH 5 citrate-phosphate buffer, where it presents its optimum activity at the "optimum pH assay", too. Its relative activity was characterized as 100% at the pH 5 citrate-phosphate buffer. The second highest enzyme activity was after its incubation with pH 8 tris-HCl buffer. BsGH48 is the most stable at its optimum pH values. BsGH48 seems to be more stable than other similar enzymes isolated from *Bacillus spp.* BICel48B is an enzyme isolated from *B. licheniformis* that can retain 65-90% of its maximum relative activity at a pH range of 4.0-6.0 (Evandro Ares de Araújo et al. 2018). BpGH48 is an enzyme isolated from an alkaliphile *B. pumilus* that presents a low activity at high pH values (Ogonda et al. 2021). *Bacillus safensis* is generally a salt-tolerant microorganism. The strain, from which BsGH48 was isolated, after its isolation from a soil sample, was tested for its cellulolytic activity by being incubated at pH 9 in a bioreactor for 4 days (P Christakopoulos et al. 1999). This strain has not been characterized but its tolerance at high pH values explains BsGH48's pH stability profile.

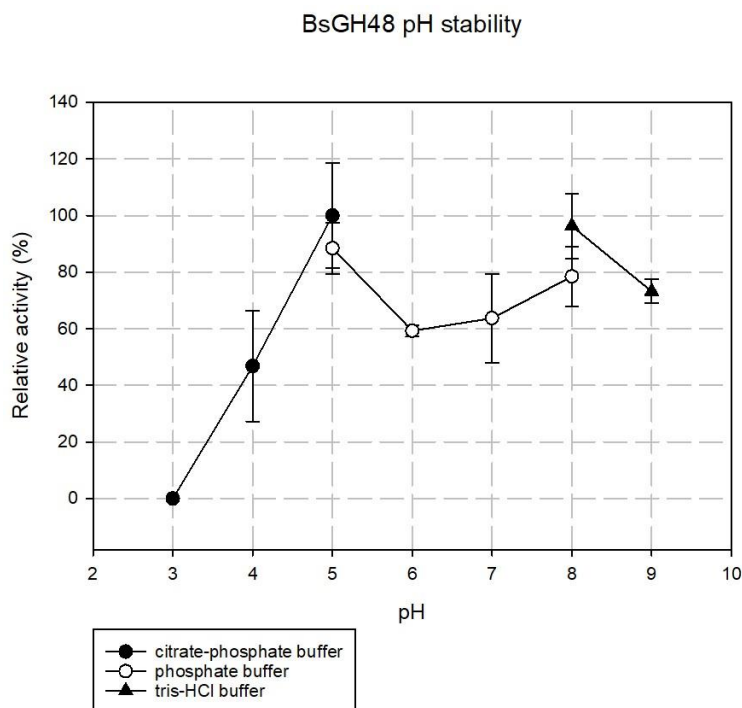


Figure 23. BsGH48's pH stability. The enzyme was incubated for 24 hours with each buffer. Then, the standard assay was conducted.

3.5. Hydrolysis products

BsGH48 hydrolyzes mainly the substrates: Avicel and PASC. In order to compare the products of the hydrolysis of these two substrates an enzymatic reaction was conducted at 55 °C, 100Mm phosphate buffer pH 5.5, for 6 hours with 100 mM of the substrate. The standard assay was altered because of the following step of this experimental procedure. The samples were then analyzed with High-performance anion-exchange Chromatography with pulsed amperometric detection (HPAEC-PAD). The data resulting from this procedure come in the form of chromatograms. As standards, the following oligo- and mono-saccharides were analyzed: glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose. Based on the retention times of each standard component the hydrolysis products were identified. The retention times of the standard components are mentioned in Table 13. Any peak presented with a retention time prior to 5 minutes belongs to the flow through of the analysis.

Table 13. The retention time of the standard components at HPAEC-PAD.

Standard Oligosaccharides	Retention Time
glucose	5.19 (±0.17)
cellobiose	8.22 (±0.16)
cellotriose	9.62 (±0.15)
cellotetraose	10.91 (±0.09)
cellopentaose	12.02 (±0.17)
cellohexaose	12.97 (±0.22)

The analysis of BsGH48's hydrolysis products did not present any clear result when Avicel was the substrate. On the contrary, cellobiose was the main product when PASC was the substrate as shown in Figure 24.

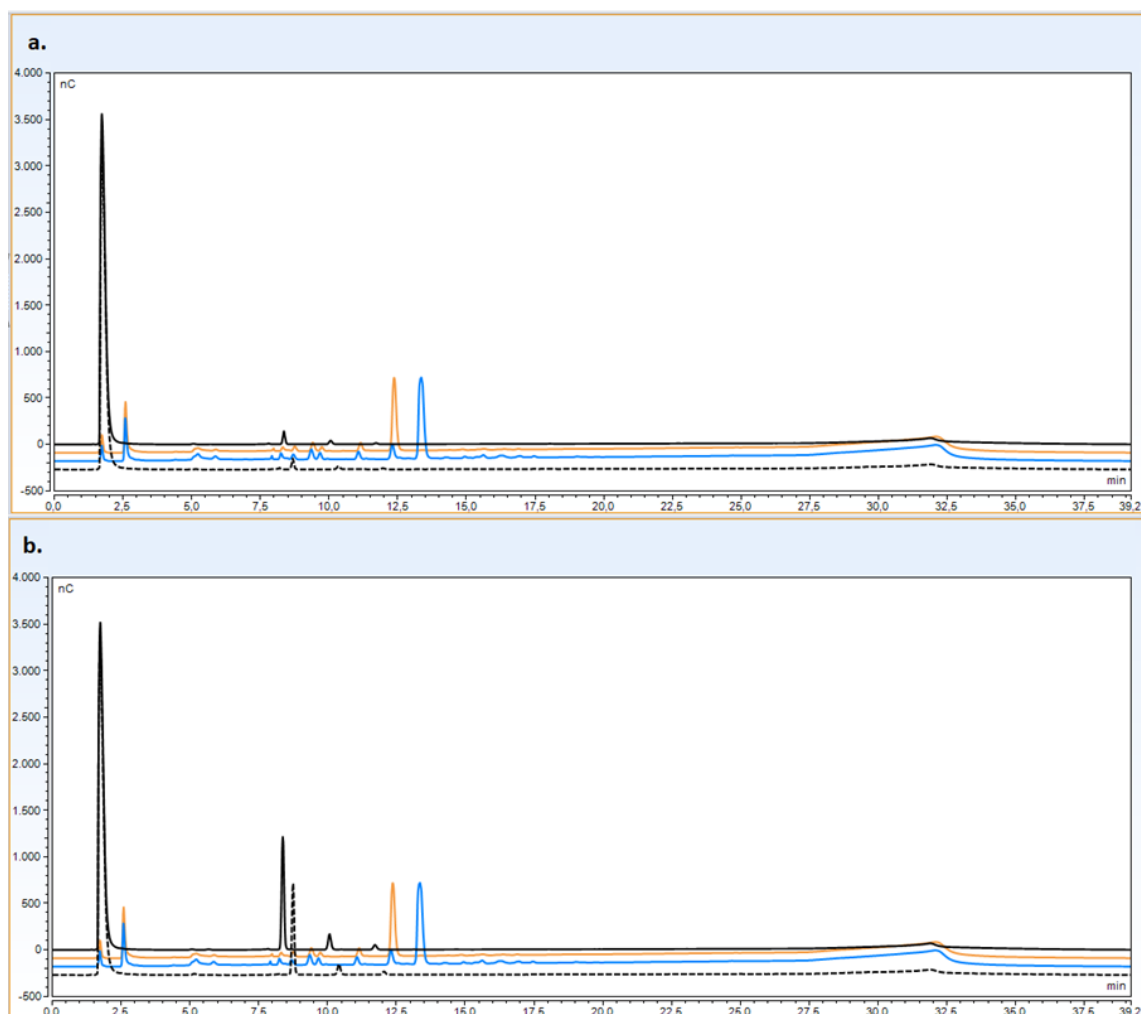


Figure 24. Chromatograms of the BsGH48's hydrolysis products. In the above chromatograms, the sample is presented with a solid black line. The blank, which is the same as the reaction sample but without the enzyme, is indicated with a black dotted line. The standard oligosaccharides, cellopentaose, and cellohexaose, are presented with orange and blue solid lines consequently. (a) Avicel was used as the substrate of this sample's reaction. (b) PASC was used as the substrate of this sample's reaction.

To measure the concentration of BsGH48's hydrolysis products the standard assay with PASC as the substrate was conducted with various time durations of the reaction: 0, 2, 4, 6, 8, 10, and 24 hours. The enzyme's concentration used in each reaction was 1 mg/mL. New standards were analyzed for this procedure. The standard components were the same as before but in different concentrations: 62.5, 125, 250, and 500 μ M.

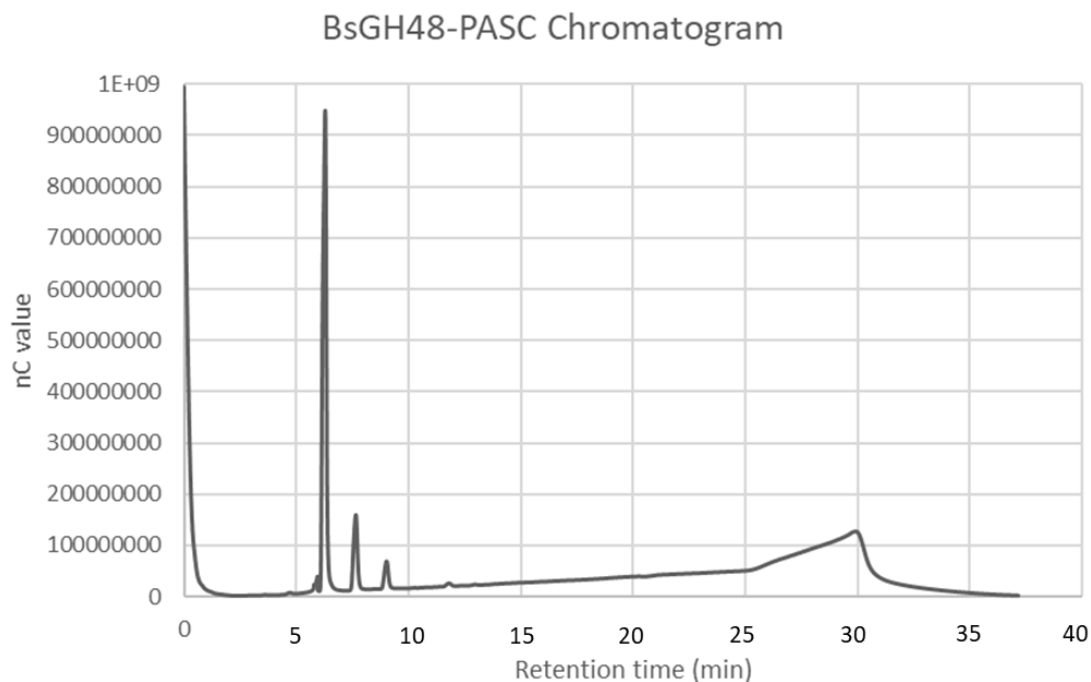


Figure 25. The chromatogram resulted from the analysis of a 10-hour enzyme reaction.

In Figure 25 the biggest peak belongs to cellobiose regarding the standards' analysis, with a retention time of 8.13 minutes. As depicted in the chromatograph there are two more peaks, one for cellotriose with a retention time of 8.38 minutes, and one for cellotetraose with a retention time of 9.77 minutes. However, their concentrations were not measurable based on the standards. Therefore, cellobiose was the only product that was produced in a measurable quantity from the hydrolysis of PASC.

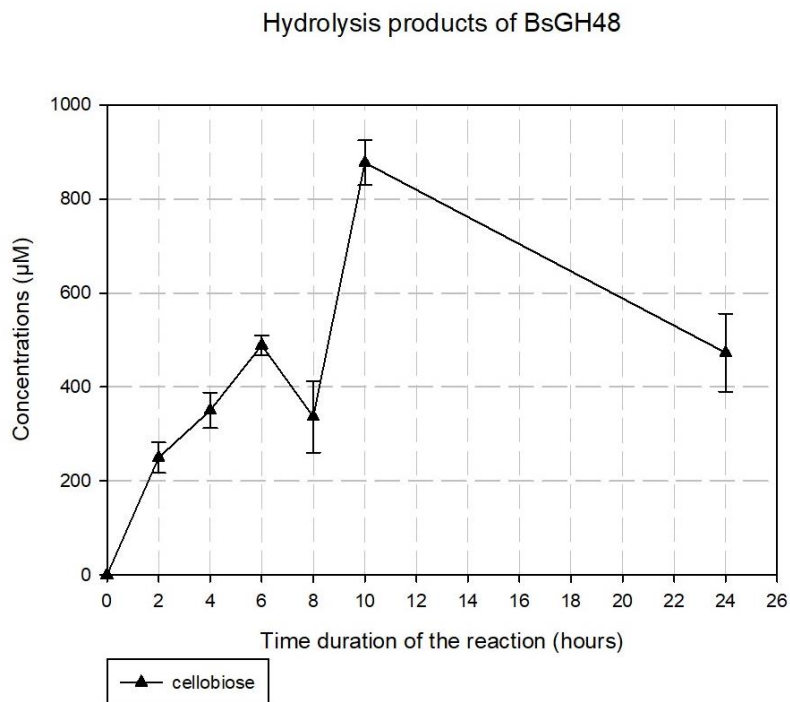


Figure 26. The concentration of cellobiose in the different enzymatic reactions.

The concentration increase of the hydrolysis product is depicted in Figure 26. Cellobiose was produced at a steady rate for the 6 first hours. At 8 hours a little decrease in the product's concentration was observed in the class of $\sim 150 \mu\text{M}$. An instant increase was presented at 10 hours of the enzymatic reaction. The concentration of the product was almost doubled. The concentration of cellobiose after 24 hours of enzymatic hydrolysis was $472,96 \mu\text{M}$. The decrease in cellobiose concentration at the 24-hour enzymatic reaction can be attributed to BsGH48 inactivation due to the enzyme's confined thermal stability. These results confirm the characterizations of BsGH48 as a β -1,4-exoglucanase or cellobiohydrolase.

4. Synergism of BsGH48 with BsGH5 and BsGH9

BsGH5 and BsGH9 are two enzymes isolated from the same strain as BsGH48, *B. safensis* (ATHUBA63). BsGH5 is a β -glucanase and BsGH9 is an endo-1,4- β -D-glucanase. Both enzymes can hydrolyze PASC. In order to study their synergistic action, it was necessary to specify each enzyme's products from the hydrolysis of PASC. Enzymatic reactions were conducted with each enzyme and 100 mM PASC as the substrate at 55°C , 100mM phosphate buffer pH 5.5, for different time durations: 0, 2, 4, 6, 8, 10, and 24 hours. The concentration of the enzyme used in

each reaction was 1 mg/mL. The conditions that were chosen are the intersection points between the optimum conditions of the three enzymes.

The hydrolysis products of BsGH5 are depicted in Figure 27 and Figure 28. BsGH5 produces numerous products and some appear in different durations of the reaction. Its products are mainly cellobiose, cellotriose, and cellotetraose. There was a small amount of glucose produced but only in a few samples.

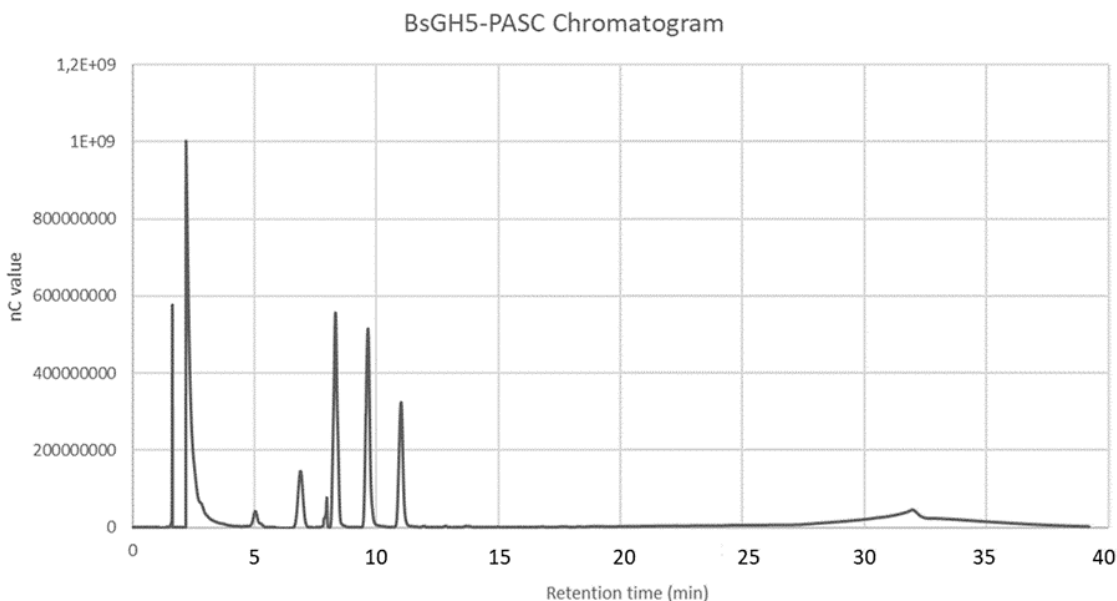


Figure 27. The chromatogram resulted from the analysis of a 10-hour enzyme reaction.

In the chromatogram, depicted in Figure 27, the biggest peak belongs to cellobiose regarding the standards' analysis with a retention time of 8.35 minutes. There are two more big peaks, one for cellotriose with a retention time of 9.68 minutes, and one for cellotetraose with a retention time of 11.03 minutes. The small peak at 5.04 retention time is glucose. The reason that it is not detected in all samples is that it is produced in a very low concentration that is not always measurable. In the chromatogram, there is another peak between glucose and cellobiose but in this experimental procedure, there was not any standard component matching this retention time.

Hydrolysis products of BsGH5

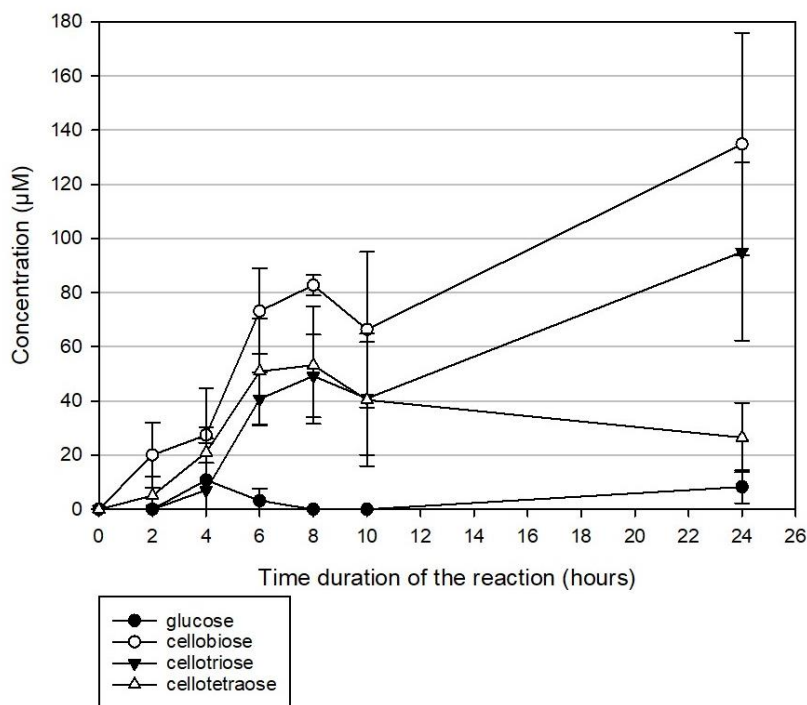


Figure 28. The concentration of the various hydrolysis products from the different time durations of the reaction of PASC by BsGH5.

As shown in Figure 28, cellotriose appears only after 4 hours of hydrolysis, while glucose only appears in two samples, the 4-hour and the 24-hour ones. The production of glucose does not appear in either the other samples or the glucose-test assays that were conducted. Cellobiose is the product that presents the biggest increase during the enzymatic reaction. Its increase appears to be steady during the hydrolysis process. Cellotriose's concentration increases simultaneously with cellotetraose's. However, in the 24-hour sample cellotriose's concentration is 3.6 bigger than cellotetraose's. At some point BsGH5 might use cellotetraose as a substrate to hydrolyze and both glucose and cellotriose are produced.

The results of the hydrolysis of PASC from BsGH9 are shown in Figure 29 and Figure 30. Glucose and cellobiose were the only products of PASC's hydrolysis. The products of BsGH9 were produced in higher concentrations than BsGH5 and BsGH48. This indicates that BsGH9's enzymatic activity is greater than the other two enzymes. In Figure 29 the two products, cellobiose, and glucose, of BsGH9's action are depicted. The biggest peak belongs to cellobiose regarding the standards' analysis with a retention time of 8.18 minutes. The other peak belongs

to glucose with a retention time of 5.09 minutes. Both products are produced in great concentrations. There are two a lot smaller peaks depicted in the chromatogram, but their concentrations were not measurable.

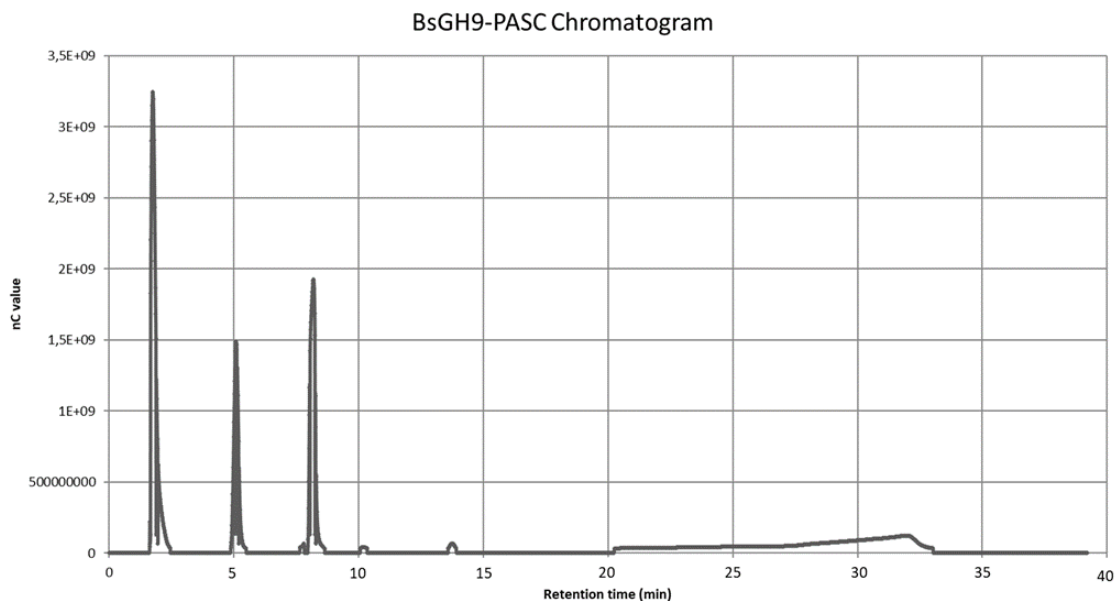


Figure 29. The chromatogram resulted from the analysis of a 6-hour enzyme reaction of PASC by BsGH9.

The increase of all the products is steady regarding the time duration of the enzymatic reaction, based on Figure 30. Cellobiose was the first product produced by the enzymatic reaction, even from the zero-hour sample. This confirms that BsGH9 is a way more active enzyme than BsGH5 and BsGH48. In the 2-hour and 4-hour samples, cellobiose is produced in a higher concentration than glucose. However, in the other samples (6h, 8h, and 24h), the ratio between glucose and cellobiose production appears to be almost steady. It is possible that PASC was completely hydrolyzed by BsGH9 in 6 hours.

The concentration of the substrate and each enzyme in the volume of the reaction is the same, and so is the time duration of the reactions of the samples. More specifically, cellobiose is produced in the highest concentration of all the enzymes. The average value of cellobiose produced at a 10-hour enzymatic reaction was 66.37 μM , 2627.47 μM , and 877.22 μM by BsGH5, BsGH9, and BsGH48 respectively. There is a great difference between the enzymatic action of these enzymes.

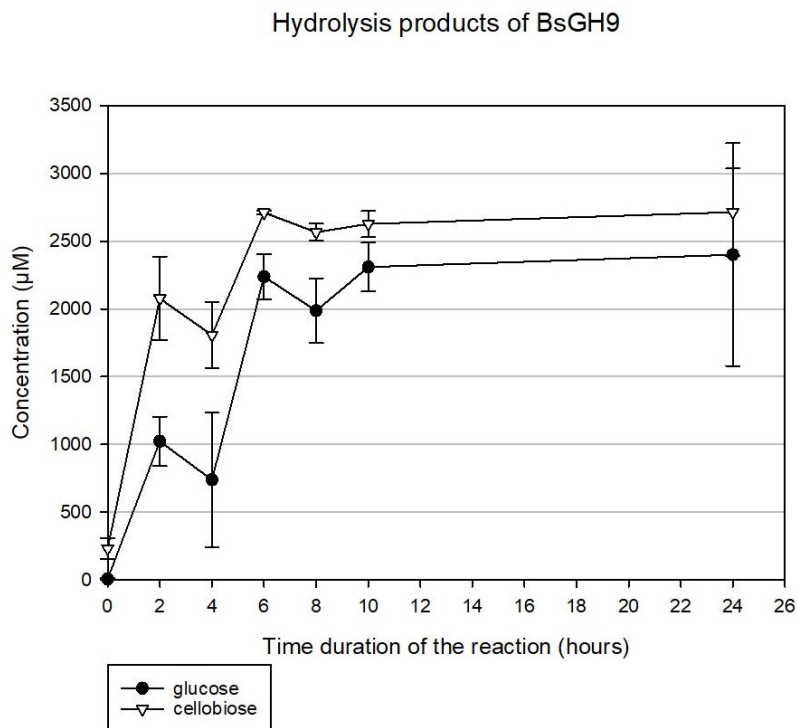


Figure 30. The concentration increase of the products from the hydrolysis of PASC from BsGH9.

After the completion of the BsGH5 and BsGH9 study, the three enzymes were paired by two, to study their synergistic action. The final enzyme concentration of the reaction was again 1mg/mL. This means that each enzyme was 2x diluted, but the final enzyme concentration remained the same. The first pair tested was BsGH5 and BsGH9 (Figure 31). The products of the BsGH5-BsGH9 synergistic reaction are glucose, cellobiose, and cellotetraose. There is no production of cellotriose as expected from the presence of BsGH5. It is possible that BsGH9 hydrolyzed cellotriose immediately after its production, and that is the reason why it does not appear in the reaction's products. Cellobiose appears in the zero-hour sample at a low concentration. This is also observed in BsGH9's zero-hour sample. In the 2-hour sample, cellobiose is produced in a higher concentration than glucose. However, the two products did not appear a great difference between their concentrations. Glucose concentration steadily and rapidly increases regarding the time duration of the enzymatic reactions. On the contrary, cellobiose concentration starts to decrease after 4 hours of enzymatic reaction. It reaches a concentration lower than 1000 μM at the 10-hour sample and remains this low at the 24-hour sample, too. This is unexpected because both enzymes produce cellobiose, but only BsGH9

produces glucose at a steady ratio regarding cellobiose. There is cellotetraose in the 10-hour sample which presents a small decrease in the 24-hour sample.

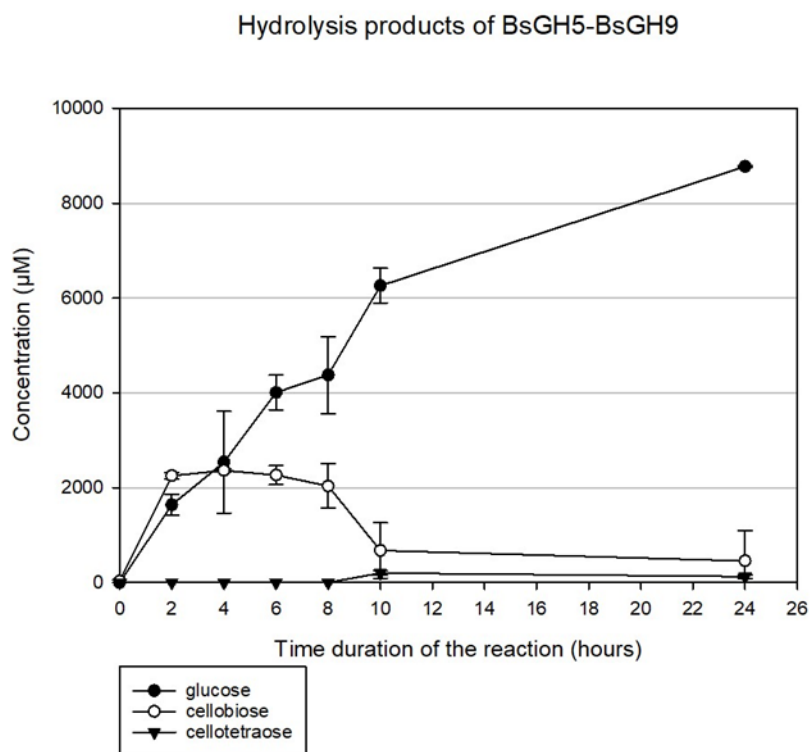


Figure 31. The results of the synergistic action of BsGH5 and BsGH9.

The second pair tested was BsGH5 and BsGH48 (Figure 32). These enzymes appeared smaller enzyme activities regarding BsGH9. The products produced by the synergistic hydrolysis were glucose, cellobiose, cellotriose, and cellotetraose. Cellobiose production is steadily increasing regarding the duration of the hydrolysis process. Its concentration after a 24-hour reaction is about 6 times bigger than other products' concentrations. This is expected since both enzymes produce cellobiose as their main hydrolysis product with PASC as a substrate. Cellotriose first appears in the 4-hour sample, and cellotetraose in the 6-hour one. Both these products indicate a small increase, with cellotriose having a slightly higher concentration than cellotetraose in all samples. Glucose is only present with a very low concentration in the 8-hour and the 10-hour samples. There is not any new product deriving from the BsGH5-BsGH48 synergism. However, the production of cellobiose and cellotriose was enhanced by the synergism. Cellotriose concentration is almost doubled from the one derived solely from BsGH5's action.

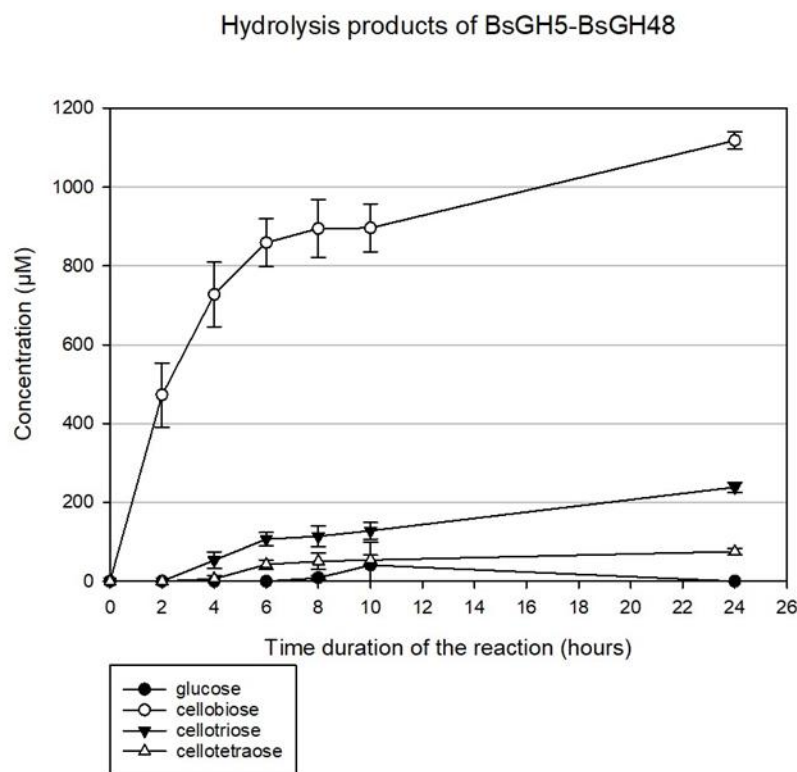


Figure 32. The results of the synergistic action of BsGH5 and BsGH48.

The third pair tested was BsGH9 and BsGH48 (Figure 33). The products derived from this synergism were glucose, cellobiose, and cellotetraose. Glucose is the product with the highest concentration. Although, it does not present a steady increase. Glucose concentration decreases in the 6-hour and 8-hour samples, but it increases abruptly in the 10-hour sample and then it decreases again at the 24-hour one. Cellotetraose appears in all the samples, but the 4-hour, 10-hour, and 24-hour ones have almost the double concentration of the rest samples. Cellotetraose appears as a product in this reaction because BsGH9 is probably occupied with the large concentration of cellulose and it was not hydrolyzed entirely. Cellobiose presents a steady increase for a 10-hour reaction, and then it starts reaching a certain plateau. When, cellobiose starts reaching this plateau at the 6-hour sample, BsGH9 probably starts hydrolyzing the cellotetraose, producing more cellobiose. This is likely the reason cellotetraose's concentration decreases in the 6-hour and 8-hour samples. Then, more cellobiose is available in the reaction mixture for BsGH9 to hydrolyze. The increased concentration of glucose in the 10-hour sample might have been due to cellobiose hydrolysis by BsGH9. Cellotetraose regains its concentration

because BsGH9 is again occupied with cellobiose. The 24-hour sample concentration might derive from the longer duration of the enzymatic reaction.

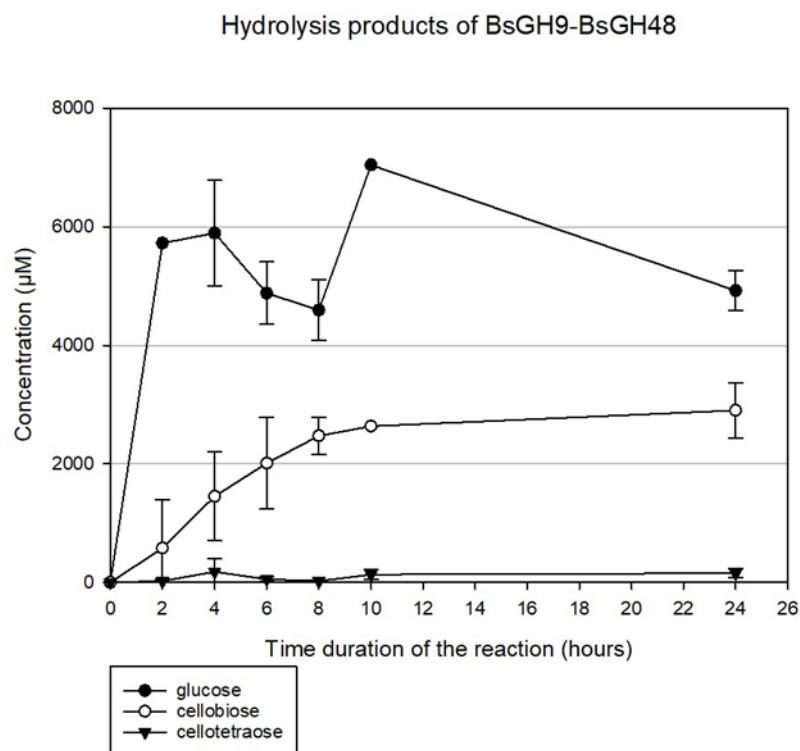


Figure 33. The results of the synergistic action of BsGH9 and BsGH48.

After examining all the possible pair-ups of these three enzymes, the synergism of all of them was tested (Figure 34). Again the final enzyme concentration was 1 mg/mL. This time each enzyme was x3 diluted. The final products of this synergism were glucose, cellobiose, and cellotetraose. Glucose and cellobiose are equally produced for the first four hours of the enzymatic reaction. Then, cellobiose remains almost steady for another 6 hours of enzymatic reaction. However, glucose in the 6-hour sample decreases, but then it surpasses cellobiose concentration for the rest of the reaction. Cellotetraose appears in the 4-hour, 20-hour, and 24-hour samples. The 4-hour sample presents a high concentration of cellotetraose. It is possible that the abrupt increase of glucose in the 4-hour sample is due to cellotetraose hydrolysis. Cellotriose only appears in extremely low concentrations, which is why it is not considered a reaction product.

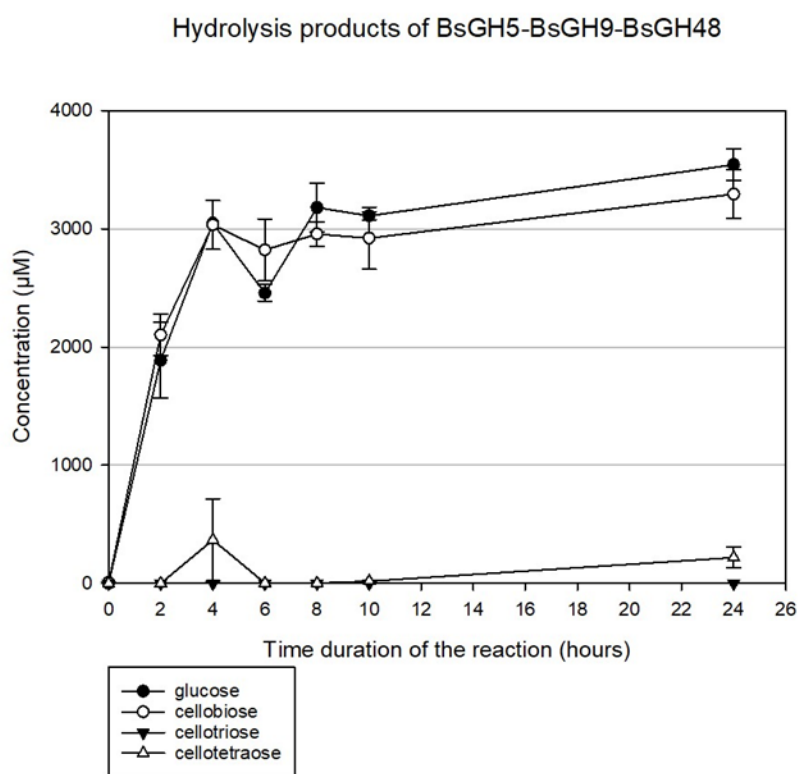


Figure 34. The results of the synergistic action of BsGH5, BsGH9, and BsGH48.

The total concentration of the products is lower when they were all included in the reaction mix, than when the enzymes are paired up.

DISCUSSION

The purpose of this thesis was to isolate, express, and characterize a β -1,4-exoglucanase or cellobiohydrolase of the GH48 protein family. Additionally, its synergistic action with an endo-1,4- β -D-glucanase of the GH9 protein family and a β -glucanase of the GH5 protein family was determined. These proteins originated from the *Bacillus safensis* (ATHUBA63) strain, which is a part of the national microbial culture bank of the National and Kapodistrian University of Athens, and it was isolated from a soil sample from Attica in 1998.

The *Bacillus safensis* (ATHUBA63) strain was cultured, and *bsgh48* was isolated via the Polymerase Chain Reaction technique. The gene of interest was inserted in the pet15b16s plasmid vector. Bacterial cells of the *Escherichia coli* DH5a strain were transformed with the recombinant plasmid. This strain is ideal for the enhancement of a plasmid vector. The enhanced recombinant plasmid vector was isolated and introduced in bacterial cells of the *Escherichia coli* BL21 strain for the production of BsGH48. This strain is a perfect choice for protein production. The production of BsGH48 was induced by IPTG. The bacterial cells were collected and sonicated, for the BsGH48 to be released in the buffer solution because the enzyme is intracellularly produced. The enzyme was purified from the cell remnants by affinity chromatography using a Ni-NTA column. Gel filtration followed to remove the imidazole, and salts of the chromatography's buffer, and also adjust the pH at 7 through buffer exchange.

A bioinformatics analysis took place, and based on the known amino acid sequence of BsGH48 valuable information was collected. The enzyme was validated as a monomer, its size was estimated at 78.6 kDa, and its 3D structure was predicted. A comparison between the known amino acid sequence of BsGH48 and other known enzymatic sequences from online databases (<https://www.uniprot.org/>). The bioinformatics analysis stated the expected results that will result from the experimental procedures that were followed.

Firstly, the molecular weight of BsGH48 was experimentally estimated with SDS-PAGE electrophoresis at ~74 kDa. The experimental value does not differ a lot from the bioinformatics prediction. Other enzymes of the GH48 family originating from *Bacillus spp.* have similar molecular weights. The BICel48 of *Bacillus licheniformis* has a molecular weight of 77.2 kDa (Evandro Ares de Araújo et al. 2018), and the BpGH48 of *Bacillus pumilus* has 79.59 kDa (Ogonda et al. 2021).

The optimum temperature of action for BsGH48 is 50 °C. There are not a lot of proteins in this family biochemically characterized to comment if this is an expected optimum temperature. It is generally close to the optimum temperature of bacterial enzymes from *Bacillus spp.* BICel48 of *Bacillus licheniformis* has an optimum temperature of action at 60 °C (Evandro Ares de Araújo et al. 2018). BpGH48 of *Bacillus pumilus* has an optimum at 55 °C (Ogonda et al. 2021), but BpumGH48 which originates from the same species presents optimum action at 37 °C (Brunecky et al. 2017b). GH48 family enzymes include mesophilic, thermophilic, and extremely thermophilic enzymes. For example, CtCelS from *Clostridium thermocellum* presents an optimum action at 70 °C (Kruus et al. 1995), and CbCelA from *Caldicellulosiruptor bescii* at 85 °C (Brunecky et al. 2013).

The thermal stability of BsGH48 was also studied at the temperature range of 40-70 °C. BsGH48 had a great sensitivity at the highest temperature tested. The half-life of the enzyme at 70 °C was 2.09 minutes. On the other hand, at the lower temperature, the enzyme indicated a half-life of 533.15 minutes. However, the inactivation of this enzyme was abrupt. The enzyme lost 40% of its activity in 1 hour of incubation at 60 °C, but it lost 85% of its activity at 5 minutes when it was incubated at 65 °C. Other characterized cellulases from the *Bacillus spp.* present a similar thermal stability to BsGH48. BICel48's enzymatic activity at 60 °C was reduced by more than 60% after a 1-hour incubation. Although, BICel48 of *Bacillus licheniphormis* lost only 20% of its activity at 50 °C after 12 hours, on the contrary BsGH48 ~20% of its activity at the same temperature at 45 minutes (Evandro Ares de Araújo et al. 2018).

From the results of the thermal stability study an Arrhenius plot was constructed, and based on that the thermal inactivation energy ($E_{(a)d}$) of BsGH48 was calculated at 159.7 ± 7.3 kJ/mole. The higher the value of the $E_{(a)d}$ is, the more thermostable the enzyme is (Saqib and Siddiqui 2018). The thermodynamic parameters of thermal deactivation of BsGH48 were estimated based on $E_{(a)d}$. The constants that were computed are the deactivation enthalpy change (ΔH^*), the Gibbs free energy of deactivation (ΔG^*), and the activation entropy change (ΔS^*). Their values are mentioned in Table 12. The ΔH^* reveals the number of noncovalent bonds in enzymes. The intensity of the thermal disruption needed for an enzyme to enter the state of its inhibition depends on the plentitude of bonds. The ΔG^* is the energy required for the enzyme to enter the denaturation phase. It is a great aggregate index of the thermal stability of an enzyme (Gouzi, Depagne, and Coradin 2012). The ΔS^* shows the disorder of the enzyme structure due to

thermal increase (Ibrahim et al. 2021). BsGH48 indicates great thermostability since the thermodynamic parameters are almost steady at the temperature range of 40-60 °C.

The optimum pH of action for BsGH48 is 5.0. Its highest activity was observed at citrate phosphate buffer pH 5.0. However, at tris-HCl buffer pH 8.0, its activity reached 96.7% of the maximum relative activity. So BsGH48 technically presents two optimum pH values. There is no reference for a cellulase having two optimum pH values, one in acidic and one in alkaline pH range. There is a bovine milk acid phosphatase that presents an optimum pH range between 4.5 and 6.0 and an additional pH optimum around 3.0 for different buffers (André et al. 2017). However, the optimum pH of action of BsGH48 agrees with other cellulases of *Bacillus spp.* BpGH48 and BICel48B also exhibit optimal activity at a pH of 5.0 (Ogonda et al. 2021; Evandro Ares de Araújo et al. 2018).

The pH stability of BsGH48 was also studied at the pH range of 3.0-9.0. The enzyme displayed great stability at pH ranging from 5.0 to 9.0 since it retains more than 80% of its relative activity, after a 24-hour incubation with each buffer tested. At pH 4 its activity dropped below 60% and at pH 3.0 it was equal to zero, after a 24-hour incubation. Its highest stability is shown at pH 5.0 citrate-phosphate buffer. Similar enzymes isolated from *Bacillus spp.* are not as stable as BsGH48. BICel48B retains 65-90% of its maximum relative activity at a pH range of 4.0-6.0 (Evandro Ares de Araújo et al. 2018). BpGH48 originated from an alkaliphile *B. pumilus* and presents a low activity at high pH values (Ogonda et al. 2021).

BsGH48 displayed a preference for Avicel and PASC as substrates. PASC was the substrate used for this study, but BsGH48's relative activity with Avicel was more than twice as high. With the other substrates the relative activity of BsGH48 was lower than 20%. These substrates are in order of priority: lichenan>filter paper>arabinoxylan>xylans. A low relative activity was presented with all the xylans. However, with nitrophenyl-pyranoside, CMC, and α -cellulose substrates, there was a null activity only with two of them. There was no reaction with β -glucan. The characterized cellulases of the 48 family, BICel48 and BpGH48, *Bacillus spp.* do not react with Avicel. They both present their highest relative activity on PASC, moderate or no activity on filter paper, CMC, and β -glucan, and no activity on soluble chromogenic oligosaccharides pNPG (Evandro Ares de Araújo et al. 2018; Ogonda et al. 2021).

The effect of some metal and two organic compounds on BsGH48 was studied. The relative activity of BsGH48 was enhanced by the majority of modulators. The highest performance of the enzyme was after its incubation with MnSO_4 buffer. Its relative activity was tripled after 30 minutes of incubation with manganese ions (Mn^{2+}). In the presence of CuCl_2 and MnCl_2 its relative activity was doubled after a 1-hour incubation. The Mn^{2+} and Ca^{2+} divalent ions enhance substantially the activity of BsGH48. However, BsGH48 indicates zero relative activity after a 30-minute incubation with FeSO_4 . Both ferrous ions, Fe^{2+} and Fe^{3+} , reduce its relative activity significantly. A reduction of BsGH48' activity was observed with the presence of CuSO_4 , with SO_4^{2-} probably the responsible ion for this decrease. BsGH48 displayed great resilience in the presence of SDS. It also remained active in the presence of EDTA, which means that BsGH48 does not require metal ions as activators.

The processivity of BsGH48 was examined with PASC as the substrate. The amount of the reducing sugars in the supernatant of the reaction was 0,0660 mg/mL, and in the sediment was 0,0003 mg/mL. Hence, BsGH48 is not a processive enzyme, which was expected since exo-cellulases produce more than 93% of the total reducing sugar in the solution (Wilson et al. 2012). The kinetic constants of BsGH48 were also studied with Avicel and PASC as substrates. These constants are K_m and $v_{\max}$. The double inverse Lineweaver-Burk plot, which quantifies more accurately the Michaelis-Menten equation of enzyme kinetics, was used for their calculation (Table 10). The K_m value with PASC as the substrate is lower than the one with Avicel. Thus, BsGH48 has a greater relation with PASC as a substrate than with Avicel. There are no cellobiohydrolases from *Bacillus spp.* of whom the kinetics constants were studied.

The hydrolysis products of BsGH48, with PASC as the substrate, were analyzed with HPAEC-PAD. BsGH48 gave only one product, cellobiose. Generally, cellobiohydrolases hydrolyze 1,4- β -d-glycosidic bonds present toward the end of the cellulose chain, releasing cellobiose and glucose. Cellobiose produced by these enzymes is eventually converted to glucose by β -glucosidases (Sinha, Sharma, and Yazdani 2023). The hydrolysis products of an endo-1,4- β -D-glucanase of the GH9 protein family (BsGH9) and a β -glucanase of the GH5 protein family (BsGH5), both originated from the same microorganism as BsGH48. The conditions of the reaction were the same for all the enzymes. BsGH9's products from the hydrolysis of PASC were cellobiose and glucose. BsGH5's products were mainly cellobiose, cellotriose, and cellotetraose. There was a small amount of glucose produced but only in a few samples. These results were expected based

on their biochemical characterization in previous studies. The hydrolysis products of BsGH9 and BsGH5 were analyzed with HPAEC-PAD, to set a backbone for the synergistic study of these three enzymes. BsGH9 hydrolyzes the β -1,4-glycosidic bond, but it is not specified which bond BsGH5 hydrolyzes. BsGH9 presented the highest enzymatic activity, with a great difference from the other two enzymes. Then, BsGH48 followed and the lowest enzymatic activity was noted by BsGH5.

The synergistic action of the three enzymes was studied by first pairing the enzymes and then including them in the same reaction mixture. The first pair tested was BsGH5 and BsGH9 (Figure 31). The products of the BsGH5-BsGH9 synergistic reaction were glucose, cellobiose, and cellotetraose. Cellotriose did not appear at the products of the reaction as expected, probably because BsGH9 hydrolyzed it immediately after its production. Interestingly, cellobiose concentration starts to decrease after 4 hours of enzymatic reaction, while the concentration of glucose increases steadily. Cellobiose is produced by both enzymes, but glucose is only by BsGH5. BsGH9 has probably hydrolyzed all the β -1,4-glycosidic bonds in a short time, leaving BsGH5 the only enzyme that produces products for the rest of the reaction time.

The second pair tested was BsGH5 and BsGH48 (Figure 32). The products derived from the synergistic hydrolysis were mainly cellobiose, cellotriose, and cellotetraose. Glucose was only present with a very low concentration in two samples. Cellobiose production was steadily increasing regarding the duration of the hydrolysis process, which was expected since both enzymes produced cellobiose. The BsGH5-BsGH48 synergism enhanced the enzymatic action of the two enzymes since cellobiose and cellotriose were produced in higher concentrations.

The third pair tested was BsGH9 and BsGH48 (Figure 33). The products produced by this synergism were glucose, cellobiose, and cellotetraose. The highest concentration in total was presented by glucose. Cellobiose had a steady increase and cellotetraose was present in all samples. BsGH9 can hydrolyze the β -1,4-glycosidic bonds randomly. The products of its action are then further hydrolyzed, therefore, cellotetraose appears in this reaction. BsGH9 was probably occupied with the large concentration of cellulose and cellotetraose was not hydrolyzed entirely.

The synergism of all the enzymes was then studied (Figure 34). The concentration of each enzyme was reduced because the total enzyme concentration of the reaction had to remain

steady. The final products of the synergism were glucose, cellobiose, and cellotetraose. Cellobiose and glucose were equally produced for the first four hours of the enzymatic reaction, but then glucose concentration surpassed cellobiose. Cellotetraose appeared only in a few samples. It is a product of the action of both BsGH9 and BsGH5, but BsGH9 can also further hydrolyze it. Hence, it is not clear why cellotetraose appeared in these samples only. The total concentration of the products was lower when they were all included in the reaction mix, than when the enzymes were paired up. This difference in the concentration products might result from the fact that each enzyme had a lower concentration in this synergistic reaction.

BsGH48 can be easily produced and isolated. It does not display optimum activity at extreme temperatures or pH values, and at the same time, it shows great stability under a variety of conditions. It is the only cellulase that presented two optimum pH values, one in acidic and one in alkaline pH range. The enzymatic activity of BsGH48 is not affected negatively by most modulators. On the contrary, its enzymatic activity is greatly enhanced by the presence of some. BsGH48 has a great specificity to its substrates and can hydrolyze microcrystalline cellulose. Few cellobiohydrolases of the 48-protein family have been biochemically characterized, so this enzyme is of great interest. Moreover, the synergism enhances the enzymatic activity of all the enzymes and the hydrolysis of phosphoric acid swollen cellulose, which indicates a huge interest in their industrial applications. Finally, it is proposed that the synergism of BsGH5, Bsgh9, and BsGH48 have to be tested based on their enzymatic activities, on cellulose raw materials, in the presence of some modulators and with the gradual addition of each enzyme.

APPENDIX

Table 14. In this table, the highly conserved tunnel-forming residues of GH48s are depicted. The residues between the two *Bacillus* species are 100% conserved. The non-conserved residues are shown in bold front. (Brunecky et al. 2017b)

<i>B. pumilus</i> (SAFR-032)	<i>C. bescii</i>	<i>T. fusca</i>	<i>C.</i> <i>thermocellum</i>	<i>C.</i> <i>cellulolyticum</i>	<i>B. safensis</i> (ATHUBA63)
His30	His	His	His	His	His51
Glu38	Glu	Glu	Glu	Glu	Glu59
Ala39	Ala	Ala	Ala	Ala	Ala60
Glu49	Glu	Glu	Glu	Glu	Glu70
Thr106	Thr	Thr	Thr	Thr	Thr127
Ala108	Ala	Ile	Ala	Ala	Ala129
Leu121	Leu	Leu	Leu	Leu	Leu142
Trp150	Trp	Trp	Trp	Trp	Trp171
Asn174	Asn	Asn	Asn	Asn	Asn195
Phe176	Phe	Tyr	Phe	Phe	Phe197
Gln177	Gln	Gln	Gln	Gln	Gln198
Thr210	Ile	Thr	Thr	Thr	Thr231
Glu212	Asp	Asp	Asp	Asp	Glu233
Gln214	Asn	Asn	Ser	Gly	Gln235
Gln219	Gln	Gln	Gln	Gln	Gln240
Arg221	Arg	Arg	Arg	Lys	Arg242
Thr223	Thr	Thr	Thr	Thr	Thr244
Asp227	Asp	Asp	Asp	Asp	Asp249
Lys267	Lys	Lys	Lys	Lys	Lys288
Tyr268	Tyr	Tyr	Tyr	Tyr	Tyr289
Trp295	Trp	Trp	Trp	Trp	Trp316
Tyr296	Tyr	Tyr	Tyr	Tyr	Tyr317
Ser298	Ala	Ser	Ala	Ala	Ser319
Trp309	Trp	Trp	Trp	Trp	Trp330
Trp311	Trp	Trp	Trp	Trp	Trp332
Ile313	Ile	Ile	Ile	Ile	Ile334
Tyr322	Tyr	Tyr	Tyr	Tyr	Tyr343
Tyr403	Tyr	Trp	Tyr	Tyr	Tyr424
Trp411	Trp	Trp	Trp	Trp	Trp432
Met414	Phe	Phe	Phe	Met	Met435
Trp417	Trp	Trp	Trp	Trp	Trp438
Asp534	Asp	Asp	Asp	Asp	Asp555
Glu590	Glu	Glu	Glu	Glu	Glu611
Arg592	Arg	Pro	Arg	Arg	Arg613
Arg682	Arg	Arg	Arg	Arg	Arg703
Trp684	Trp	Trp	Trp	Trp	Trp705

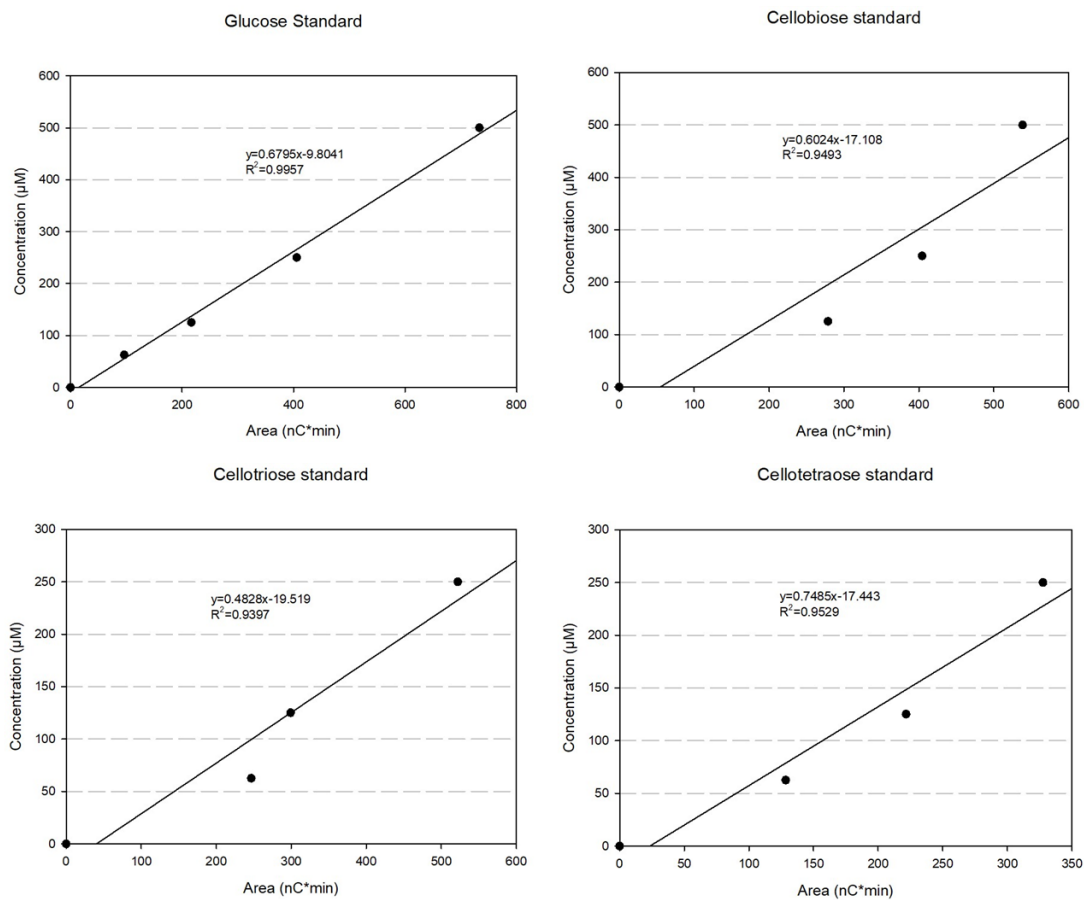


Figure 35. The standards used in the hydrolysis products analysis.

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