Production, characterisation, and application of *Beauveria bassiana* SAN01 β-glucosidase

Buka Welcome Magwaza

Student Number: 21805819

Submitted in fulfilment for the Degree of

Master of Applied Science

in

Biotechnology

Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

March 2025

SUPERVISOR: Prof Santhosh Kumar Kuttan Pillai

CO-SUPERVISOR: Dr Ayodeji Amobonye

DECLARATION

I, Mr. Buka Welcome Magwaza (21805819), and Prof Santhosh KK Pillai and Dr Ayodeji Amobonye hereby declare the following that in respect of the dissertation entitled "Production, characterisation and application of *Beauveria bassiana* SAN01 β-glucosidase"

1. The research described in this thesis was carried out in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa, under the supervision of Prof Santhosh KK Pillai and Dr Ayodeji Amobonye.

2. As far as I ascertain:

a) no other similar dissertation exists.

b) the only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

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3. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

26-02-2025

Signature of student **Date**

03 March 2025

Signature of supervisor **Date**

03 March 2025

Signature Co-supervisor **Date**

ACKNOWLEDGEMENTS

First and foremost, praise and thanks to almighty God for protecting me throughout this research. I would like to express my deepest gratitude to my major supervisor, Prof. Santhosh Pillai for his invaluable, guidance, encouragement, sacrifices and enthusiasm throughout the the project. I could not have imagined a better supervisor and mentor for my study.

Many thanks to my co-supervisor Dr Ayodeji Amobonye for his great assistance, sacrifices, and guidance in completing the project as well as Dr Prashant Bhagwat for his useful suggestions and support.

I am grateful to my research group members and my colleagues who provided me with the most pleasant and convenient working environment.

I am thankful to the Department of Biotechnology and Food Science for giving me this opportunity to pursue my studies.

Special thanks to my parents, whole family, and my best friend for their endless support, from my heart thank you.

Finally, I would like to appreciate National Research Foundation (NRF) for financial assistance.

DEDICATION

This work is dedicated to my late mother, Judith Ntombiyakhe Khumalo, my aunt, Zothile Khumalo, my brother, Thami Khumalo, and my little brother, Sizwe Khumalo for their endless support and love.

Research outputs

Published Articles

1. **Magwaza, B**., Amobonye, A. and Pillai, S. 2024. Microbial β-glucosidases: recent advances and applications. *Biochimie*, 225: 49-67. https://doi.org/10.1016/j.biochi.2024.05.009

2. **Magwaza, B**., Amobonye, A., Bhagwat, P. and Pillai, S. 2024. Hyper β-glucosidase producer *Beauveria bassiana* SAN01—optimisation of fermentation conditions and evaluation of saccharification potential. *Biomass Conversion* and *Biorefinery*, pp.1-13. https://doi.org/10.1007/s13399-024-05866-x

3. **Magwaza, B**., Amobonye, A., Bhagwat, P. and Pillai, S. 2024. Biochemical and *in silico* structural properties of a thermo-acid stable β-glucosidase from *Beauveria bassiana*. *Heliyon*, 10 (7) e28667. https://doi.org/10.1016/j.heliyon.2024.e28667

Conference presentation

Buka Magwaza, Ayodeji Amobonye, Prashant Bhagwat, Santhosh Pillai. A novel β-glucosidase from the hyper-producer *Beauveria bassiana* SAN01. South African Society for Microbiology, Stellenbosch University, Western Cape, South Africa, 17 – 20 September 2023.

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Abbreviations

β-glucosidase	BGL
Central Composite design	CCD
Glycosyl hydrolase	GH
gds	gram per dry substrate
HPLC	High-performance liquid chromatography
kDa	Kilodalton
PDA	Potato dextrose agar
PB	Plackett-Burman
pNP	p-nitrophenol
pNPG	4-nitrophenol β-D-glucopyranoside
SmF	Submerged fermentation
SSF	Solid-state fermentation
TLC	Thin-layer chromatography

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Abstract

Beauveria bassiana is a popular entomopathogenic fungal endophyte that is used industrially as a biocontrol agent. It has been noted to be non-pathogenic to humans, animals and plants and their ability to utilise various agro-residues for its metabolism has been exploited for the production of some lignocellulosic enzymes. Previous studies have mostly focused on the production of key lignocellullose hydrolysing enzymes, however, little is known about the ability of *B. bassiana* to produce accessory enzymes such as β-glucosidase which also aid in lignocellullose breakdown. Hence, this study was aimed at investigating the production, the biochemical characteristics, and the potential application of a β-glucosidase from the strain designated as *Beauveria bassiana* SAN01. For these aims to be achieved, the production parameters of β-glucosidase were optimised using a statistical approach. Furthermore, to enhance the evaluation of the biochemical properties and potential industrial application of the enzyme, it was purified to homogeneity using salt precipitation, and chromatography.

The preliminary screening of seven agricultural residues showed that the haulm of Bambara- an underutilised African legume- supported the highest β-glucosidase production, hence, statistical optimisation of enzyme production was performed using this biomass as the sole carbon source. The three-level statistical optimisation experiments resulted in a ~5.36-fold increase in β-glucosidase production from the unoptimised level of 132.71 U/mL to 711 U/mL, under optimal conditions (Bambara - 57 g/L, KCl - 302 mg/L, NaCl -154 mg/L, agitation -150 rpm, and incubation time - 223 h). Ammonium sulphate precipitation followed by dialysis and gel filtration chromatography were used to purify β-glucosidase produced by *B. bassiana* SAN01 to homogeneity. The purified β-glucosidase was demonstrated to have a specific activity of 496 U/mg and a molecular mass of ~116 kDa by SDS-PAGE; its activity pattern was also confirmed via in-gel zymography using 4-methylumbelliferyl-β-D-glucopyranoside as the substrate. The enzyme activity was recorded to be optimal at pH 5.0 and 60°C and the enzyme also displayed significant thermal stability from temperatures 30-50°C, retaining almost 60% of its activity at 50°C after 4 h of incubation. Subsequently, the potential of *B. bassiana* SAN01 β-glucosidase as an accessory enzyme in lignocellulose saccharification was demonstrated by its effectiveness in the hydrolysis of cellobiose converting more than 90% of the substrate to glucose. Finally, some structural insights were gained into the enzyme using a computational approach. The *in silico* prediction of the enzyme revealed that it has an isoelectric point of 5.59, that it was hydrophilic and thermostable. The modelled 3D structure of *B. bassiana* β-glucosidase confirmed that it belongs to the GH 1 family and the model was

validated by the presence of ~ 96% of its amino acid residues in the favoured region of the Ramachandran plot. The docking of the enzyme with cellobiose and 4-nitrophenol β-D-glucopyranoside demonstrated the significant affinity of both substrates to the enzyme while revealing its most probable active site. Results from this study demonstrate *B. bassiana* as a hyper-producer of β-glucosidase as the production level in this study is one of the highest ever recorded for an entomopathogenic fungi; thus the study highlights the immense potential of *B. bassiana* in the processing of lignocellulosic biomass to biofuels.

1 CHAPTER ONE

INTRODUCTION AND REVIEW OF LITERATURE

Biochimie 225 (2024) 49–67

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Microbial β-glucosidases: Recent advances and applications

Buka Magwaza, Ayodeji Amobonye, Santhosh Pillai *

Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, P. O. Box 1334, Durban, 4000, South Africa

ARTICLE INFO

Article history:
Received 22 February 2024
Received in revised form
5 April 2024
Accepted 6 May 2024

Handling Editor: O.A. Dontsova

ABSTRACT

The global β-glucosidase market is currently estimated at ~400 million USD, and it is expected to double in the next six years; a trend that is mainly ascribed to the demand for the enzyme for biofuel processing. Microbial β-glucosidase, particularly, has thus garnered significant attention due to its ease of production, catalytic efficiency, and versatility, which have all facilitated its biotechnological potential across different industries. Hence, there are continued efforts to screen, produce, purify, characterize and evaluate the industrial applicability of β-glucosidase from actinomycetes, bacteria, fungi, and yeasts. With this rising demand for β-glucosidase, various cost-effective and efficient approaches are being explored to discover, redesign, and enhance their production and functional properties. Thus, this present review provides an up-to-date overview of advancements in the utilization of microbial β-glucosidases as "Emerging Green Tools" in 21st-century industries. In this regard, focus was placed on the use of recombinant technology, protein engineering, and immobilization techniques targeted at improving the industrial applicability of the enzyme. Furthermore, insights were given into the recent progress made in conventional β-glucosidase production, their industrial applications, as well as the current commercial status-with a focus on the patents.

Part of this chapter has been published: **Magwaza, B**., Amobonye, A. and Pillai, S. 2024. Microbial β-glucosidases: recent advances and applications. *Biochimie*, 225: 49-67. https://doi.org/10.1016/j.biochi.2024.05.009

1. Introduction

Beta-glucosidases (E.C.3.2.1.21) are essential component of the cellulase enzymatic system which also comprises the endoglucanases (E.C.3.2.1.4), and the exoglucanases (E.C.3.2.1.91). Beta-glucosidases (BGL), which can also be referred to as β-D-glucoside glycohydrolase, catalyse the hydrolysis of the glycosidic bond within carbohydrate moieties to produce non-reducing terminal glycosyl residues, glycosides, and oligosaccharides (Mól et al., 2023a). This terminal reaction has been identified as the rate-limiting step in the hydrolysis of cellulose to glucose. The enzyme, which is quite ubiquitous in nature, plays various biochemical, physiological, and nutritional roles in organisms across all kingdoms (Singhania et al., 2017). The various biological functions of the enzyme have since been translated into various applications in the industry which include the hydrolysis of lignocellulose for biofuel production, hydrolysis of glycosides in fruit juices and wines for improved aroma, synthesis of bioactive aglycones from glucoside conjugates, as well as the production of alkyl glucosides that are useful ingredients of cosmetics and detergents (Godse et al., 2021).

The industrial importance of these enzymes has ensured that they form a significant part of the global enzyme market. Statistics revealed that BGLs constituted a significant share (11%) of the global enzyme market in 2021, a share that was projected to continue to rise in subsequent years (360i Research, 2021). It is believed that the projected increase in demand for this enzyme is mainly driven by the gradual shift to cleaner energy alternatives, especially biofuels. BGLs play a critical role in making bioethanol production both economically viable and environmentally sustainable by facilitating the efficient hydrolysis of cellulose into fermentable sugars (Ramachandran et al., 2022). Thus, similar to other biotechnologically important biocatalysts, various strategies have since been devised to enhance their production, stability, applicability, and affordability, all being crucial factors in the industry. Most of these strategies have been developed in the last two decades and they include but are not limited to, recombinant enzyme production, enzyme immobilisation, and various protein engineering approaches.

For instance, various studies have been carried out to clone and express BGL genes in different vectors with the aim of increasing production levels and/or the ease of purification (Zada et al., 2021b, Liang et al., 2019). Recently, a strain of *Clostridium cellulolyticum* was engineered using CRISPR-Cas9n technology to produce a recombinant BGL with ~7-fold increased activity and enhanced cellulose degradation, thus increasing its suitability in a consolidated biorefinery (Tao et al., 2023). Besides the energy industry, the application of a recombinant

BGL from *Lactobacillus brevis*, which possessed significant acid and ethanol tolerance, was also demonstrated in wine-making (Li et al., 2022). However, despite the undeniable role of BGL in the quest for clean energy and environmental sustainability, which are both core mandates of the United Nations Sustainable Goals (which are covered under SDG 7 & 11, respectively), the significant advances made in the production and application of the enzyme has not been fully covered in recent times.

Thus, in addressing the gaps highlighted above, this literature reviews recent research and commercial milestones on the production and industrial applications of BGL from microbial sources.

1.1 *Beauveria bassiana*, an entomopathogenic fungal endophyte

Beauveria bassiana is mainly identified as an entomopathogenic fungus that antagonize, parasitize and kills numerous insects, including *Coleoptera, Diptera*, *Hemiptera*, *Lepidoptera*, as well as arthropods belonging to the subclass *Acari* (Harith-Fadzilah et al., 2021). *B. bassiana* has been employed as a biocontrol agent for several pests including the pine caterpillar (*furnacalis*), peach aphid (*Myzus persicae*), pine sawyer (*Bursaphelenchus xylophilus*), and corn borer (*Ostrinia furnacalis*) across global locations, owing to the aforementioned capability. This fungus has gained recognition in the North America, Europe, Asian pacific, Latin America, Middle East, and Africa over the past decades as an effective biological control. The global market of *B.bassiana* is projected to reach USD 195.6 million by 2027, from USD 65 million in 2022, at a CAGR of 17.0% during 2021-2027.

B. bassiana mode of action occurs through the attachment of the fungus's conidia to the host's cuticle via hydrophobic interactions, followed by germination. Subsequently, *B. bassiana* develops a germ tube, which exhibits the ability to infiltrate the outer layer of the host organism, known as the cuticle, using a combination of mechanical force and the action of enzymes that degrade components of the cuticle, including chitinase, lipase, and proteases (Bhadani et al., 2021). Upon reaching the host's body cavity, the fungal structures undergo growth as individual cells known as blastospores, which proceed to invade the immune system of insects. These blastospores derive nutrition from the haemolymph and release toxins that ultimately result in the demise of the insects. The fungus penetrates the cuticle from the interior to the exterior, facilitating the development of conidia. Upon dispersal, these conidia initiate fresh infections after the death of the insect (Fig. 1.1).

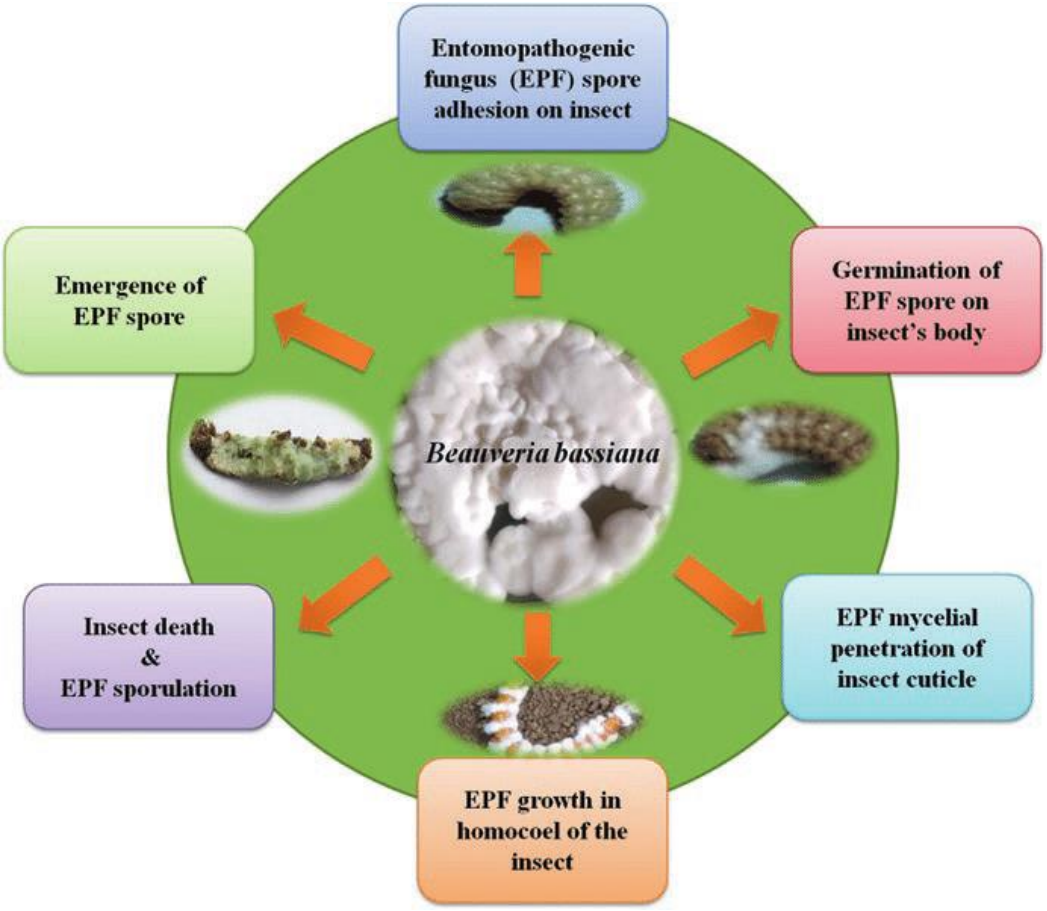

Figure 1.1: Overview of the infection life cycle of *B. bassiana* during insect attack (Sharma et al., 2020a)

In addition to its entomopathogenic capabilities, *B. bassiana* has also been acknowledged as an endophyte by colonizing different plants or crops such as pecan, cassava roots (Greenfield et al., 2016), *Capsicum annuum* (Saragih, 2019), *Solanum lycopersicum* (Barra-Bucarei et al., 2020), and *Phaeseolus vulgaris* L (Afandhi et al., 2019). This fungus colonises these plants to enable pest, pathogens, and harsh conditions resistance as well as to promote their growth (Mantzoukas et al., 2021, Qin et al., 2021). For instance, *B. bassiana* was used in integration pest management to suppress the effect of aphid in *Capsicum annum* (Jaber and Araj, 2018). Moreover, *B. bassiana* was observed to promote the growth of *Gossypium hirsutum* by suppressing the population of Helicoverpa zea (Lopez and Sword, 2015). Recently, *B. bassiana* was observed to stimulate the growth of rice under salt stress conditions (Akter et al., 2023). In another recent study performed by Wang et al. (2023), *B. bassiana* displayed insecticidal activity in tomatoes through the production of plant secondary metabolites such as α-solanine, 5-O-caffeoylshikimic acid, clerodendrin A, and peucedanin respectively. Therefore, due to

these abilities, the market for *Beauveria bassiana* in the agricultural sector is expected to rise in the coming years.

1.2 *Beauveria bassiana* as a source of biocatalysts

Studies have shown that *B. bassiana* has also been employed as whole-cell biological catalyst (Amobonye et al., 2020). The fungus produces chitinase, lipases, and proteases as the major enzymes to degrade insect cuticle which are made up of proteins, lipids, and polysaccharide chitin respectively (Pelizza et al., 2020), however, there is a variety of other industrial enzymes that have been noted to be synthesised by this fungus under both liquid and solid fermentation.

1.2.1 Chitinase

Chitin, a linear polymer composed of β-1, 4-N-acetylglucosamine units, is widely recognised as the second most abundant polymer on earth, surpassed only by cellulose. The presence of this substance has been seen in the exoskeletons of various creatures, including fungi, insects, crustaceans, and yeast. According to Liu et al. (2019a), chitinase is responsible for the hydrolysis of chitin into chitiooligomers. *B. bassiana* exhibits the production of chitinases, which serve multiple physiological roles including the destruction of the insect's cuticle composed primarily of chitin, defence against other fungi inhabiting the same ecological niche, hyphae fusion, and autolysis (Oyeleye and Normi, 2018). Previous studies have investigated the screening of chitinase from *B. bassiana* by submerged fermentation and solid substrate fermentation methods, utilising various substrates such as colloidal chitin, wheat bran, and shellfish as complex carbon sources. According to Elawati et al. (2018), it has been discovered that colloidal chitin is the most effective carbon source for chitinase synthesis in *B. bassiana*.

The maximum production of chitinase by *B. bassiana* through submerged fermentation (SmF) and solid-state fermentation (SSF) has been reported to occur within a period of four to five days from the initiation of incubation. The five chitinase enzymes derived from *B. bassiana* was observed to possess theoretical pI values between 4.81-7.94 (Bhagwat et al., 2021).To date, limited research has been conducted on the characterisation of chitinase derived from *B. bassiana.* However, a study by Dhar and Kaur (2010) has demonstrated that chitinase derived from *B. bassiana* has an optimal temperature range of 30 to 48°C, with an estimated molecular weight of approximately 70 kDa. The chitinase gene Bbchit1 from *B. bassiana* has been successfully cloned in many host organisms, including *Escherichia coli* and *Pichia pastoris*, to enhance production levels (Fan et al., 2007, Kim et al., 2023).

1.2.2 Lipases

Lipases are enzymes that facilitate the hydrolysis of long-chain fatty acids and glycerol, resulting in the production of ester linkages (Devi et al., 2020). The catalytic function of lipases is only activated by the presence of an oil-water contact. *B. bassiana* predominantly synthesises lipases to enzymatically break down the hydrophobic epicuticle of insects (Gonçalves et al., 2020). This epicuticle consists of a diverse combination of lipids, long-chain alkenes, esters, and fatty acids. Consequently, lipases primarily catalyse the hydrolysis of ester bonds present in lipoproteins, fats, and waxes located within the interior region of the insect integument (Xu et al., 2018). Microbial lipases have garnered significant attention across a range of industries, including food, leather, detergent, cosmetics, and pharmaceutical sectors, owing to their remarkable versatility in catalysing diverse reactions (Chandra et al., 2020).

Lipases have been previously produced by various strains of *B. bassiana* by both submerged fermentation and solid-state fermentation methods, exploiting diverse fat waste materials as a carbon source (Gonçalves et al., 2020, Zibaee et al., 2011). In numerous studies, it has been noted that the presence of triglycerols, such as olive oil, stimulated the synthesis of lipase (Gonçalves et al., 2020). Zibaee et al. (2011) reported that the peak production of lipase by *B. bassiana* was 144 h and 166 h of incubation during submerged fermentation. The lipases derived from *B. bassiana* exhibit a range of pH preferences, including acidic (Zibaee et al., 2011) and alkaline conditions (Sugahara and Varéa, 2014). Additionally, *B. bassiana* lipase has been observed to be mesophilic and thermophilic, For instance, *Beauveria* lipase obtained by Zibaee et al. (2011) displays optimal temperature ranges of 35 to 50°C, whereas *B. bassiana* lipase obtained by Vici et al. (2015) and Sugahara and Varéa (2014) display optimal temperature at 60°C. The molecular weight of lipase derived from *B. bassiana* has ranged from 25 to 64 kDa (Kim et al., 2023, Pérez et al., 2019).

1.2.3 Proteases

Proteases are a class of hydrolytic enzymes that catalyse the cleavage of peptide bonds inside proteins, resulting in the formation of smaller peptides and individual amino acids. According to Walsh (2015), Microbial protease enzymes constitute 60% of the industrial enzymes that are deemed crucial for the infective process. Entomopathogenic fungi are capable of synthesising protease enzymes, specifically serine-protease, which facilitate the degradation of proteinaceous substances present in the epicuticle of insects. This degradation process occurs subsequent to the action of lipase and chitinase enzymes, as shown by Zhang et al. (2008). The production of protease enzymes from *B. bassiana* through submerged fermentation (SmF) has

been previously reported in numerous studies (Akrich et al., 2023, Borgi et al., 2016, Mancillas-Paredes et al., 2019). The highest protease level (2000 U/mL) ever reported was obtained on *B. bassiana* P2 strain by Borgi et al. (2016). Another significant protease production level of 280.72 U/mL was obtained in another *Beauveria* sp. after 7 days of incubation under liquid fermentation (Rao et al., 2006). Moreover, further optimisation of protease level on the 30 L airlift reactor yielded 283.84 U/mL from the same strain, which is in close agreement to the level obtained in shake flask experiments respectively.

The molecular weights of proteases derived from *Beauveria* spp have been shown to exhibit a wide range, spanning from a lower molecular weight of 32 kDa (Borgi et al., 2016) to a higher molecular weight of 101 kDa (Mancillas-Paredes et al., 2019). The application of protease enzymes derived from various species of *Beauveria* has been explored in different contexts. For instance, the serine protease obtained from *B. bassiana* has demonstrated efficacy as a detergent, specifically in the removal of blood stains from cotton fabric. Additionally, it has been observed to possess the capability to degrade melanised feathers (Borgi et al., 2016). The proteases derived from *Beauveria* MTCC 5184 was observed to be alkaline in nature with an isoelectric point of 9.3 (Shankar et al., 2011).

1.2.4 Other industrial biocatalysts produced by *Beauveria bassiana*

These enzymes constitute the most extensive category of industrial enzymes, widely utilised in various applications within the field of biotechnology including food and beverages, pharmaceutical, feed industry, and bioenergy sector etc (Marar and Garg, 2020). The enzymes encompassed in this category consist of amylase, cellulases, β-glucosidase, and pectinase, etc.

1.2.5 Amylase

Amylases are widely recognised as one of the enzymes most extensively employed in industrial applications. According to Gopinath et al. (2017), the process of starch hydrolysis involves the enzymatic action of amylases, which break down starch molecules into monomers or smaller polymers, including glucose and dextrin. The utilisation of this enzyme has been observed in the food industry to convert starch into the sugar syrup. Additionally, it has garnered attention in other industries such as detergent, pharmaceutical, textile, and paper (Gopinath et al., 2017). Several studies have been conducted to investigate the capacity of *B. bassiana* to synthesise amylase when provided with various carbon substrates. Numerous studies have reported the production of amylase enzyme from different strain of *Beauveria bassiana* (Amobonye et al., 2020, Mngguang and Shenghua, 2002, Martins et al., 2014). However, the most noteworthy

finding was the attainment of an amylase activity level of 13151.82 U/mg from *B. bassiana* SG8702 (Mngguang and Shenghua, 2002). *B. bassiana* SG8702 amylase was observed to be stable at pH 4.0 and 40°C (Mngguang and Shenghua, 2002), whereas amylase from *B. bassiana* SAN01 was reported to be stable at pH 6.0 and 35°C. In addition, *B. bassiana* SAN01 amylase was used in trial studies for the clarification of pear juice and it was able to breakdown starch molecules present in the juice into small monomers and subsequently improve the clarity of the juice respectively (Amobonye et al., 2020).

1.2.6 Cellulases

Cellulose, a linear and crystalline polymer, represents the most abundant polymer found on Earth. It is composed of repeated sugar units of glucose that are connected by β-1, 4 glycosidic bonds. Cellulose, hemicellulose, and lignin are the primary components of plant biomass, comprising roughly 40-50%, 23-35%, and 15-20% respectively (Li et al., 2021). Cellulases represent a class of enzymes that play a crucial role in the hydrolysis of cellulose. Cellulose has garnered significant interest within the energy sector due to its potential as a sustainable alternative raw material for the manufacturing of bioethanol and chemicals. This recognition derives from the recent acknowledgment of cellulose as a viable substitute for fossil fuels, which are known to have detrimental environmental impacts. Cellulase has been widely applied in several industries such as pulp and paper, textiles, and food processing (Singh et al., 2021). The production of cellulase by *B. bassiana* has been conducted using submerged fermentation using different substrates such as carboxymethylcellulose, wheat bran, etc. The thermophilic nature of a cellulase derived from *B. bassiana* B1432 was reported in a study by Petlamul et al. (2017) with the enzyme displaying an optimal temperature of 80°C. Under stability test the enzyme retained about 55% of its initial activity (3.59 U/ml) after 50 min of incubation at this optimum temperature. Recently, endo and exo-cellulase were obtained by Alves et al. (2020) from another *B. bassiana* strain under solid-state fermentation.

1.2.7 Xylanases

Xylanases are a type of glycoprotein that plays a crucial role in the process of depolymerizing xylan, breaking it down into simpler monosaccharides and xylooligosaccharides. The utilisation of xylanase in various industrial applications has been observed, such as its use in the food industry for the clarity of fruit juice, enhancement of shelf life, and improvement of the texture of baked products. Xylanase enzymes have been employed in the pulp and paper industries to enhance the brightness of pulp, hence facilitating the creation of high-quality paper (Sharma et al., 2020b). Xylanase has garnered significant attention in the energy sector

due to its use in the conversion of the xylan structure found in lignocellulosic materials into xylose sugar. This xylose sugar serves as a carbon source for the synthesis of bioethanol (Chaudhary et al., 2021). Recent studies have indicated that *B. bassiana* exhibits the ability to effectively use several types of lignocellulosic biomass for the production of xylanase . The xylanase enzymes derived from *Beauveria* spp have been observed to possess an acidic nature, demonstrated by their optimal pH range of 6-6.5. The xylanase derived from *B. bassiana* SAN01 was shown to have an optimal pH of 6.0, as reported by Amobonye et al., (2021). Similarly, the xylanase from *Beauveria* MTCC 5184 (More, 2017) and *B. bassiana* B14532 had optimal pH values of 6.0 and 6.5 respectively, according to Petlamul and Boukaew (2019). The optimal temperature range for the *Beauveria* strain has been reported to be between 45°C and 55°C. The application of xylanase derived from *B. bassian*a SAN01 was also demonstrated with successful results in the deinking process of paper (Amobonye et al., 2021).

1.2.8 Pectinase

Pectin can be obtained in all plants across the globe (Haile and Ayele, 2022). Pectinases (EC 3.2.1.15) are responsible for the hydrolysis of pectin into monogalacturonic acid molecules. Based on their mode of action, they are classified into seven classes including, pectinesterase (EC 3.1.1.11), polygalacturonase (EC 3.2.1.15), galacturan 1, 4-α-galactouronidase (EC 3.2.1.67), exopoly-α-galactouronosidase (EC 3.2.1.82), endopectate lyase (EC 4.2.2.2), exopectate lyase (EC 4.2.2.9) and endopectin lyase (EC 4.2.2.10) (Shrestha et al., 2021). These enzymes make up 25% of the global food enzyme market (Ruiz et al., 2012). Therefore, there was a growing interest in the production of pectinase enzyme in last past years due to their numerous applications in food processing. The production level of 51 U/mL of polygalacturonase has been recently highlighted in *B. bassiana* SAN01 (Amobonye et al., 2020). The polygalacturonase which was used for the clarification of juice pear exhibited a maximum activity at pH 7.0 and 35°C. Furthermore, this enzyme exhibited significant stability between 25-50°C, retaining about 50% of the actual activity after 120 min incubation (Amobonye et al., 2020).

1.3 Microbial sources of β-glucosidase

β-glucosidases are a diverse group of enzymes produced by a wide variety of microorganisms and higher organisms such as animals and plants. These organisms produce BGL for various biological functions including nutritional acquisition, defence, and ecological associations, however, most organisms utilize the enzyme for the hydrolysis of oligosaccharides to glucose, which is the most utilizable form of carbon (Mól et al., 2023a). Microbial sources of BGL are

more feasible for industrial applications as microbes can be easily grown for enzyme production, furthermore, they can be easily manipulated through genetic and protein engineering (Singh and Chhatpar, 2011). Thus, BGLs from algae, bacteria (including actinomycetes), and fungi (including yeasts) across various ecosystems including soil, plants, and water bodies, have been identified and studied for their biochemical properties and industrial relevance (Table 1).

There is an inexhaustive list of bacteria that have been studied for their ability to produce BGL; notable among them are *Bacillus subtilis, B. lincheniformis, B. halodurans, Bifidobacterium bifidum, Cellulomonas fimi, Escherichia coli, Clostridium thermocellum, Lactobacillus acidophilus, L. brevis*, *Pseudomonas fluorescens, Thermobifida fusca, Thermoanaerobacter ethanolicus* and *Pseudomonas* spp. (Ahmed et al., 2017). However, the *Bacillus* genus has been noted to be the most prolific BGL producer, as shown by different studies on highly functional BGLs from *B. altitudinis* JYY-O2 (Yang et al., 2022), *B. licheniformis* (Chen et al., 2017a) and *B. subtilis* (Bagudo et al., 2014). For instance, a thermostable BGL from *B. altitudinis* JYY-02 was recently demonstrated to hydrolyse geniposide in gardenia fruits to genipin, thus catalysing an important step in gardenia blue production (Yang et al., 2022). Lactic acid bacteria such as *Lactobacillus harbinensis* and *Pediococcus pentosaceus* were also noted to secrete BGLs with various industrial potentials (Liang et al., 2022).

Other bacterial classes, especially the actinomycetes, have also been shown to be effective BGLs producers. Actinomycetes are a class of bacteria that are considered unique enough to be discussed as an individual group, especially because of their industrial significance. In addition to their acclaimed antibiotic-producing capabilities, actinomycetes are also known to secrete a wide range of industrially important enzymes (Uhoraningoga et al., 2021, Aytaş et al., 2023). Thus, actinomycetes such as *Jiangella alba* DSM 45237 (Aytaş et al., 2023), *Streptomyces griseus* (Uhoraningoga et al., 2021), *Thermomonospora* spp. and *Nocardia* spp. (Samuel et al., 2022) have been shown in different studies to produce BGL. However, the industrial applicability of actinomycetes BGLs, especially in biomass saccharification, has not been well evaluated as more focus has been placed on BGLs from other bacterial species and fungi. In general, it was also observed that despite the huge potential of bacterial BGLs (judging from their relative thermostability and wide pH tolerance), their relatively low production levels and typical intracellular localization limit the feasibility of their real-time utilization.

Fungi, especially filamentous fungi, are considered the most viable sources of BGLs due to their higher production capacity and ease of recovery from their extracellular enzyme pool. Filamentous fungi are naturally inclined to the degradation of lignocellulosic biomass in different ecosystems; hence, their remarkable BGL activities are not far-fetched (Kovács et al., 2022). Recently, BGL was sourced from various filamentous fungi, including *Aspergillus* sp. YDJ14 (Srivastava et al., 2019b), *Coniophora puteana* (Zhou et al., 2023b), *Chaetomella* spp (Singh et al., 2023a) and *Microdochium nivale* (Zhou et al., 2023b). It was observed that *A. niger* and *Trichoderma ressie* are the most commonly used fungi for commercial BGL production due to their high production levels; for example, Novozyme188 from *A. niger* (Chauve et al., 2010) and Cellic® CTec3 (Novozymes) from *T. Ressie* (da Costa et al., 2018).

However, several fungal BGLs are known to be limited by their narrow pH range and reduced stability at temperatures above 50°C, thus, affecting their utilization in industrial processes with high temperatures and high pH conditions. The BGL production potential of non-filamentous fungi, including yeasts like *Candida glabrata* (Han et al., 2023), *Hanseniaspora uvarum* BF345, *Meyerozyma guilliermondii* NM218 (Gao et al., 2022), *Issatchenkia terricola* SLY-4, *Pichia kudriavzevii* F2-24, *Metschnikowia pulcherrima* HX-13 (Liao et al., 2022) and *Wickerhamomyces anomalus C4* (Liu et al., 2020), have also been demonstrated. This is quite remarkable as yeasts, such as *Saccharomyces cerevisiae* and *Komagataella* spp., are effective in fermenting simple sugars to ethanol; therefore, their concomitant production of BGL would ensure that they can circumvent the glucose feedback inhibition, hence, increasing their applicability in one-pot synthesis (Ahmed et al., 2017).

Like most enzymes, the biochemical properties of microbial BGLs are majorly dictated by their natural environments and the physiological conditions of the microbial source. In this regard, mesophilic and thermophilic microbes would typically produce BGLs with optimum temperatures that fall within 20-45ºC and 45-70ºC respectively. Furthermore, fungi, which are majorly acidophilic, have been observed to produce BGLs that are most active at a pH range of 3.0-6.0. Hence, understanding the biochemical properties of specific microbial BGL is believed to be necessary for the efficient application of the enzyme in various industrial sectors, as well as facilitating the design of enhanced enzymes via protein engineering. Thus, many studies, including recent ones, have been devoted to the purification of microbial BGLs and as well as their subsequent biochemical characterisation (Table 1.1).

Table 1.1: Biochemical characterisation of β-glucosidase from different microbial sources

Microorganism	**pH optima**	**Temperature optima (°C)**	**Molecular weight (kDa)**	**Activators**	**Inhibitors**	**Reference**
Alteromonas sp. L82	7.0	40	54	$Ca2^{+}$, K^{+}, DTT	Zn^{2+}, Cu^{2+}, Co^{2+} and SDS	Sun et al. (2018)
Aspergillus chevalieri	5.5	45	43.4	NR	NR	Senba et al. (2023)
Aspergillus sp. YDJ14	4.0	60	100	NR	NR	Oh et al. (2018)
Bacillus licheniformis	7.0	60	53.4	Zn^{2+}, Mn^{2+}, Mg^{2+}	Cu^{2+} and Ca^{2+}	Chen et al. (2017a)
Bacillus subtilis	6.0	60	53	Mn^{2+}, Fe^{2+}	Hg^{2+}, Ag^{2+} and Cu^{2+}	Chamoli et al. (2016)
Bacillus tequelensis BD69	5.0	50	54.35	NR	NR	Raza et al. (2020)
Chaetomella sp. BBA70074	6.0	70	170	Zn^{2+} and Fe^{2+}	NR	Singh et al. (2023a)
Coptotermes formosanus	5.0	50	45	$Mn^{2+}$and Mg^{2+}	NR	Gutierrez-Gutierrez et al. (2023)
Dekkera bruxellensis	5.0	60	43	Mg^{2+}	Hg^{2+}, SDS and EDTA	Kuo et al. (2018)

Dictyoglomus turgidum	7.0	80	50	Tween 20, Triton X-100	$Zn^{2+,}$ $Mn^{2+,}$ and Cu^{2+}	Fusco et al. (2018)
Exiguobacterium sp. DAU5	7.0	45	52	Ca^{+}	Hg^{2+} and Cu^{2+}	Chang et al., (2011)
Hot spring metagenome (archaea)	6.5	90	57	Guanine hydrochloride, Triton X-100, Cr^{2+}	CTAB, Tween-80 and SDS	Schröder et al. (2014)
Humicola grisea var. *thermoidea*	6.0	50	57	NR	Fe^{2+}, Cu^{2+} ,Zn^{2+} and Al^{3+}	Benoliel et al. (2010)
Jeotgalibacillus malaysiensis	7.0	65	52	Ca^{2+}, $Mn^{2+,}$ K^{2+}	Co^{2+}, Zn^{2+}, SDS, EDTA,	Liew et al. (2018a)
Jiangella alba DSM 45237	6.0	40	NR	Ca^{2+},and Co^{2+}	Ag^{2+}	Aytaş et al. (2023)
Kluyveromyces marxianus	5.0	60	64	Mg^{2+} , K^{+}	Zn^{2+}, Ba^{2+}, Cu^{2+}	Su et al. (2021)
Malbranchea pulchella	6.0	50	93	NH_4F	Hg^{2+}	Monteiro et al., (2020)
Metschnikowia pulcherrima.	4.5	28	NR	Cu^{2+}	NR	Mateo Tolosa (2023)
*Microbulbifer s*p. ALW1	4.5	40	65	Mg^{2+}, Ba^{2+} Tween 20, Triton X-100	Zn^{2+}, $Cu^{2+}$and SDS	Jiang et al. (2021)

Myceliophthora thermophila	5.0	55	100	$Co^{2+}$and Triton X-100	Mn^{2+} and Zn^{2+}	Dadwal et al. (2023)
Myceliophthora thermophila M.7.7	5.0	60	200	Ba^{2+}	Cu^{2+} and Fe^{2+}	Bonfa et al., (2018)
Neofusicoccum parvum strain F7	4.0	65	65	n.a	$Zn^{2+,}$ Cu^{2+} Mn^{2+} and Fe^{2}	Singh et al. (2023b)
Neofusicoccum parvum strain F7	4.0	60	65	NR	$Ca^{2+}$and Mn^{2+}	Singh et al. (2023b)
Penicillium simplicissimum	5.2	60	126	Mn^{2+}	Cu^{2+},$Co^{2+}$and Fe^{3+}	Bai et al. (2013)
Proteus mirabilis VIT117	9.0	37	50	NR	NR	Mahapatra et al. (2016)
Rhodotorula oryzicola	6.0	65	52	NR	NR	de Araujo Ribeiro and Assis (2023)
Saccharomonospora sp. NB11	7.0	40	51	Tween 20	$Zn^{2+}$and Hg^{2+}	Zada et al. (2021a)
Thermoanaerobacterium thermosaccharolyticum DSM 571	6.4	70	52	$Mn^{2+}$and Fe^{2+}	$Al^{3+}$and Cu^{2+}	Pei et al. (2012)
Thermoclostridium stercorarium	5.0	65	86	NR	Ethanol	Zeng et al. (2023)

Thermococcus radiotolerans DSM-15228	5.0	80	50	Mn^{2+}	NR	Albalawi et al. (2023
Trichoderma harzianum	4.0	50	NR	NR	$Ca^{2+}$and Mn^{2+}	Sun et al. (2022)
White rot fungus	6.0	37	70	$Ca^{2+,}$and Mg^{2+}	Ni^{2+} and Cu^{2+}	Binh et al. (2022)

**NR- not reported

1.3.1 Production of microbial β-glucosidases

Over the years, the natural propensity of microbes to secrete BGLs has been harnessed for enzyme production under controlled laboratory or industrial conditions. Thus, the controlled production of BGLs has been carried out using bacteria, yeast, and fungi while utilizing different simple and complex substrates as carbon sources. While fungi readily utilize complex substrates, especially agricultural residues such as corn cob, forage palm, sugarcane bagasse, rice straw, wheat bran, and wheat straw, for BGL production, bacteria are more inclined to utilize simple sugars such as glucose, lactose, cellobiose, for the same purpose (Srivastava et al., 2019b). Furthermore, the microbial production of BGLs can either be carried out under either submerged (SmF) or solid-state fermentation (SSF) conditions. Both of these techniques possess several advantages as well as some disadvantages which will be further highlighted in this section.

1.3.1.1 Submerged fermentation

The advantages of better process control and handling have been associated with the production of various enzymes, including BGL, under SmF conditions (Singhania et al., 2010). As summarised in Table 2, this mode of fermentation has been found to be quite efficient for BGL production by most bacteria (including actinomycetes) (Aytaş et al., 2023) and some fungi including yeasts (Singh et al., 2023b, de Araujo Ribeiro and Assis, 2023). Interestingly, some bacteria including *Bacillus subtilis, B. halodurans* C-125, *B. licheniformis,* and *Pseudomonas lutea* have been demonstrated to produce BGLs while growing on substrates such as paddy straw, lactose, glucose, and sugarcane bagasse during SmF. While *B. halodurans* showed a crude BGL activity of 95 IU/mL using lactose (Naz et al., 2010), *B. licheniformis* was observed to produce 45 IU/mL of BGL when grown on glucose (Yao et al., 2016).

Although bacteria are more suited for fermentation under submerged conditions, fungal BGL production has also been demonstrated severally under such conditions. For example, *Penicillium* spp, *Aspergillus* spp, and *Trichoderma* spp were able to produce BGLs under the SmF system while utilizing different substrates. In the study by Alarid-García et al. (2022), *A. Niger* CDBB-H-175 was reported to utilize maltose to produce 2954 U/mL BGL after 31 h incubation. Recently, *Penicillium* sp. FSDE15 was able to synthesize BGLs while growing on different carbon sources such as wheat bran, sugarcane bagasse, and sugarcane straw under SmF (de Castro Coêlho et al., 2021). Non-filamentous fungi such as *Candida peltata* and *Komagataella phaffii* have also been highlighted to produce BGL at significant levels while growing in liquid cultures containing simple carbon sources such as glucose, sorbitol, and

xylose (Amer et al., 2017). Many recent studies have now been focused on the optimisation of the BGL production parameters under submerged conditions via statistical approaches and artificial machine learning. For instance, significant production of BGL (812.86 U/mL) was achieved during SmF by *A. versicolor* exploring response surface methodology (Huang et al., 2021). Artificial neural network–genetic algorithm on the other hand assisted in increasing the production of *Penicillium oxalicum* BGL by 260% (Li et al., 2021).

Table 1.1: Production of β-glucosidase by different microorganisms under submerged fermentation

Microorganism	**Temp (°C)**	**pH**	**Substrate(s)**	**Yield**	**Reference**
Aspergillus terreus MS105	35	4.8	Cellulose	0.161 IU/mL	Sohail et al., (2016)
Aspergillus niger	30	6.0	Wheat bran, corn steep liquor	9.37 IU/mL	Abdella et al. (2014)
Aspergillus terreus MS105	35	4.8	Sigmacell	0.161 IU/mL	Sohail et al., 2016
Aspergillus japonicus VIT-SB1	20	6.0	Wheat bran	10.3 U/mL	Singh et al. (2023c)
Aspergillus niger ITV-02	31	5.7	Sugarcane bagasse	10.23 U/mL	Infanzón-Rodríguez et al. (2023)
Bacillus licheniformis	60	7.0	Glucose + sucrose	45. 5 IU/mL	Yao et al. (2016)
Bacillus subtilis CCMA 0087	36.6	3.64	Coffee pulp	22.59 IU/mL	Srivastava et al. (2019a)
Bacillus halodurans C-125	45	8.0	Lactose-induced Luria broth	95 IU/mL	Naz et al. (2010)
Bacillus stercoris	40	8.0	Wheat bran and orange peel	2.89 U/mg	Ur Rahman et al. (2023)
Candida peltate NRRL Y-6888	50	5.0	Glucose + xylose + sucrose	1.5 IU/mL	Kooloth Valappil et al. (2019)
Komagataella phaffii	30	7.5	Sorbitol	3.8 U/mL	Batra et al. (2014)
Kluyveromyces marxianus	35	5.5	Okra	144 IU/mL	Su et al. (2021)
Micrococcus antarcticus	25	6.	Cellobiose	289 IU/mL	Fan et al. (2011)
Neofusicoccum parvum strain	30	6.0	Casein + glycerol	2.5 U/mL	Singh et al. (2023b)
Penicillium citrinum NAF5	30	6.0	*Parthenium hysterophorus* weed	2.48 U/mL	Kumar et al. (2022)
Penicillium funiculosum NCIM 1228	29.84	5.88	Wheat bran	NR	Chavan et al. (2023)
Penicillium sp LM101	60	6.0	Carboxymethyl-cellulose (CMC)	0.058 IU/mL	Santa-Rosa et al. (2018)

Pseudomonas lutea	30	8.0	Sugarcane bagasse	23.29 IU/mL	Tiwari et al. (2016)
Rhizopus oryzae	18	6.0	Water hyacinth	137.32 IU/mL	Karmakar and Ray (2011)
Rhodotorula oryzicola	28	NR	Cellobiose	18.4 U/mg	de Araujo Ribeiro and Assis (2023)
Trichoderma atroviridae	30	6.2	Pretreated willow	11.70 IU/mL	Kovács et al. (2008)
Trichoderma harzanium	28	5.5	CMC	0.92 IU/mL	Ahmed et al. (2009)
Talaromyces amestolkial	70	4.0	Glucose	1.8 IU/mL	Méndez-Líter et al. (2018)
Thermomyces dupontii	40	5.	Wheat bran	37 U/mL	Nisar et al. (2020)
Trichodema harzanium	28	5.5	Glucose	0.05 IU/mL	Ahmed et al. (2009)
Trichoderma asperellum LYS1	30	NR	Corn stalk	1.8 U/mL	Mou et al. (2023)

**NR- Not reported

1.3.1.2 Solid-state fermentation (SSF)

SSF is fermentation with minimal free water activity, however, the growth substrate contains enough moisture to sustain microbial metabolism and growth, hence, SSF is believed to better mimics the natural growth conditions of most filamentous fungi compared to SmF (Hansen et al., 2015). In general, *Aspergillus*, *Penicillium, and Trichoderma* species are the most prolific organisms for BGLs production while utilizing low, inexpensive substrates under SSF conditions, thus highlighting their huge potential for BGLs production for commercial use (Singh et al., 2023c). Several agro-industrial residues such as aspen wood, corncob, forage palm, paddy straw, sorghum, sugarcane bagasse, rice straw, and wheat bran have been utilised as carbon sources for BGLs production by different microbes during SSF (Table 1.3.).

However, it was deduced that forage palm and wheat bran are among the best carbon sources for the production of fungal BGLs. For example, a high production level of 937.05 U/g - which is one of the highest ever reported for BGL under SSF- was obtained while *Penicillium Roquefort* ATCC 10110 utilized forage palm as the primary growth substrate (das Neves et al., 2020). In the same vein, *Trichoderma* sp. RCK65 showed significant production of BGL (144.1 U/g) when wheat bran was used as the carbon source (Chakraborty et al., 2016), while *Lichtheimia ramose* also produced a high amount of BGL (274 U/g) with the same substrate (Garcia et al., 2015). A mixture of substrates has also been observed to support the production of BGL at significant levels; for instance, *A. niger* SH3 grown on a combination of wheat bran and wheat straw gave a BGL yield of 540.57 IU/g (Tiwari et al., 2015), Similarly, a high production level of 820 IU/g was also observed for *A. awamori* when grown on pineapple crown leaves and wheat bran mixture (Nishida et al., 2018). A few bacterial species have also been shown to produce BGLs under SSF, although at lower levels when compared to fungi. For example, *Bacillus* sp. B1, *Bacillus* sp. B2 and *Brevibacillus* sp. B3, which were all isolated from termite gut, were demonstrated to produce ~ 2.3, 3.4, and 5.1 U/g of BGL while utilizing sawdust as a carbon source under SSF conditions (Kamsani et al., 2016).

Table 1.2: Production of β-glucosidase by different microorganisms under solid-state fermentation

Microorganism	**Temp (°C)**	**pH**	**Substrate(s)**	**Yield**	**Reference**
Aspergillus niger SH3	30	4.8	Wheat bran: wheat straw	540.57 IU/g	Tiwari et al. (2015)
Aspergillus awamori	28	4.5	Pineapple crown leaves, wheat bran	820 IU/g	Nishida et al. (2018)
Aspergillus flavipes	40	5.0	Wheat bran	17.0 U/g	Dina and Thankamani (2023)
Aspergillus flavus ITCC 7680	30	4.8	Pretreated cotton stalk	96 IU/g	Singh et al. (2017)
*A*spergillus *terreus* MS105	35	4.8	Balled mild cellulose	0.56 IU/mL	Sohail et al. (2016)
Aspergillus niger	30	6.0	Breadfruit seed hull	210.1 U/g	Osaro-Matthew et al. (2023)
Aspergillus sp. DHE7	28	5.5	Jojoba meal	81.3 ± 4.2 U/g	El-Ghonemy (2021)
Chrysoporthe cubensis	28	5.0	Wheat bran	1.30 U/g	Dutra et al. (2023)
Consortium (*Pleurotus ostreatus* and *A. niger*)	45	4.0	Sorghum and wheat bran	54.9 U/g	Martins et al. (2021)
Fusarium chlamydosporum HML278	30	NR	Sugarcane bagasse	20.6 U/mg	Qin et al. (2020)
Irpex lacteus	30	4.8	Corn stover	11.1 U/g	Xu et al. (2009)
Malbrenchea cinnamonea	40	7.0	Sorghum straw	234.18 U/g	Mahajan et al. (2016)
Monascus sanguineus	60	2.5	Jack fruit seed	3.8 IU/g	Dikshit and Tallapragada (2014)
Penicillium expansum MDFS2	30	4.5	Rice bran	2.21 U/g	Sharifzadeh et al. (2019)
Penicillium roqueforti ATCC 10110	23	5.0	Forage palm	935.07 IU/g	das Neves et al. (2020)
Thermoascus auranticus RCKK	45	5.0	Wheat bran	23.31 IU/g	Jain et al. (2015)
Trichoderma asperellum UC1 and *Rhizopus oryzae* UC2	32	12.0	Oil palm leaves	131.76 U/g	Ezeilo et al. (2022)
Trichoderma sp. RCK65	30	4.5	Wheat bran	144.1 U/g	Chakraborty et al. (2016)
Trichoderma viride GIM	55	5.0	Banana peel	3.01 IU/g	Sun et al. (2011)

**NR- Not Reported

1.3.1.3 Statistical optimisation

The optimisation of fermentation conditions is of paramount importance as these factors have a substantial impact on the yield of the products. The conventional method of optimisation, commonly referred to as One Variable at a Time (OVAT), involves altering a single variable while holding all other elements constant. However, the OVAT approach is deemed inefficient for optimisation due to its disregard for the interplay between the various parameters that are, in fact, interdependent (Infanzón-Rodríguez et al., 2023). Furthermore, OVAT is both labour-intensive and costly, requiring a significant amount of time and resources. Consequently it often falls short in identifying the most optimal conditions for that particular process (Singh et al., 2023c). Response Surface Methodology (RSM), on the other hand, is a statistical technique that has been proven highly efficient in optimising fermentation conditions as well as other production processes across various industries (Mehmood et al., 2021, Kusuma et al., 2021). This method is particularly advantageous as it accounts for the interaction of several factors, hence reducing the number of tests required for analysis (Abuhena et al., 2022).

Various steps are involved in the RSM approach. Firstly, problem identification: the utilisation of RSM is typically appropriate when there is a need to determine the specific scenario in which several input factors have the potential to impact the quality attributes of products or processes. Secondly, the determination of factor levels through screening experiments: it is advisable to use a monitoring method to ascertain the factors that exert a substantial influence on interest responses. Next is the identification of the independent variables (factors) which can be manipulated and altered separately, selection of the appropriate experimental design, selection of a regression model either using first and second order polynomial, treatment of data mathematical, verification of the fitted model using standard techniques such as residual analysis, prediction error sum of squares (PRESS) and analysis of variance (ANOVA) to evaluate the accuracy between dependent and independent variables, graphical presentation of the model equation using 3D surface contour plot, prediction of optimal operating conditions, optimisation of the model, and finally validation of model respectively (Reji and Kumar, 2022).

Response Surface Methodology includes first-order designs such as Placket-Burman design (PBD), which is used to screen the most significant factors, however, it does not consider the interactions between the factors. Thus, second-order designs such as Central composite design (CCD) and Box–Behnken design (BBD) can determine the independent variable sand their effect on the response. In the context of Central Composite Design (CCD), the central points

refer to the points located in the centre of the design space. Additionally, the design includes factorial points, which are arranged symmetrically on the coordinate system's axes relative to the central point. Furthermore, axial points are also incorporated into the design, and they are symmetrically positioned concerning the central point (Beg and Rahman, 2021). Central composite designs (CCDs) offer several advantages in sequential trials since they often enable the extension of previous factorial evaluations by including axial and central points. (Pandey et al., 2019). Box and Behnken (1960) proposed a methodology that involves defining three levels for each component. These levels are derived from a specific subset of factorial combinations inside the 3k factorial designs. The sequence of numbers and the sequential analysis of the impact of different design parameters can be conducted using these models, while maintaining constant values for the other elements, to study the initial factors respectively (Pandey et al., 2019).

RSM has been extensively used for the optimisation of BGL production from various sources. For instance, RSM was recently used for the optimisation of six factors (incubation time, pH, incubation temperature, agitation, casein, and glycerol) for BGL production from a newly isolated *Neofusicoccum parvum* strain F7. The results obtained from this study showed that 12 days of fermentation, 20°C, 175 rpm, 0.5% glycerol and 1.5% casein, and pH 6 were the optimal conditions for maximum production of BGL, increasing from (1.6 U/mL) of (OVAT) to (2.5 U/mL) by RSM respectively (Singh et al., 2023b). Similarly, Huang et al. (2021) optimised the fermentation conditions for BGL production from *Aspergillus versicolor* using PBD and CCD revealing that wheat bran 15 (g/L), oatmeal 15 (g/L), KCl 5 (g/L), and rotation speed 170 rpm are optimal for maximum BGL production; a 14.4-fold increase was recorded compared to the unoptimised level. PPB and BBD were also recently employed by Infanzón-Rodríguez et al. (2023) for the optimisation of BGL production from *Aspergillus niger* ITV-02. The fermentation parameters such as 20.69 g/L of delignified sugarcane bagasse, 0.24% (v/v) of Tween 80, and pH 5.67 were observed to be optimal for BGL production, with a 47.4% increase observed with respect to the production level before optimisation using statistical response. In addition to the use of statistical approaches for optimisation of BGL production, other computational approaches such as artificial neural learning has also been exploited for BGL optimisation. For example, artificial learning such as Artificial Neural Network-genetic algorithm was observed to increase the production of *Penicillium oxalicum* BGL by 260%. (Li et al., 2021).

1.3.1.4 Valorisation of lignocellulosic biomass

Lignocellulosic biomass is considered a viable option for sustainable renewable resources due to its widespread availability and underutilization on a global scale. Lignocellulosic biomass encompasses a range of materials including agricultural waste, forest residues, organic municipal solid waste, dedicated energy crops, and industrial waste from wood, paper, and pulp companies (Saravanan et al., 2022). Lignocellulosic biomass, a type of organic polysaccharide, consists of three primary constituents: cellulose, hemicelluloses, and lignin, however, percentage of the constituent polymers may vary depending on the types of feedstocks. For instance, sugarcane bagasse contains about 40-45% cellulose, 30-35% hemicellulose, and 20-30% lignin while wheat straw contains about 33-40% cellulose, 20-25% hemicellulose and 15-20% lignin respectively (Baruah et al., 2018). Lignin exhibits intricate interconnectivity throughout lignocellulose biomass assembly, rendering them resistant to microbial and enzymatic deconstruction, hence, impeding the conversion of biomass into products with enhanced value (Rajak and Banerjee, 2015). Subsequently, a comprehensive understanding of the diverse features associated with recalcitrance and the chemical structure of polymers is crucial for the effective functioning of biomass-based bio refineries. Consequently, various pretreatment methods have been devised to overcome these limitations (Fig. 1.2).

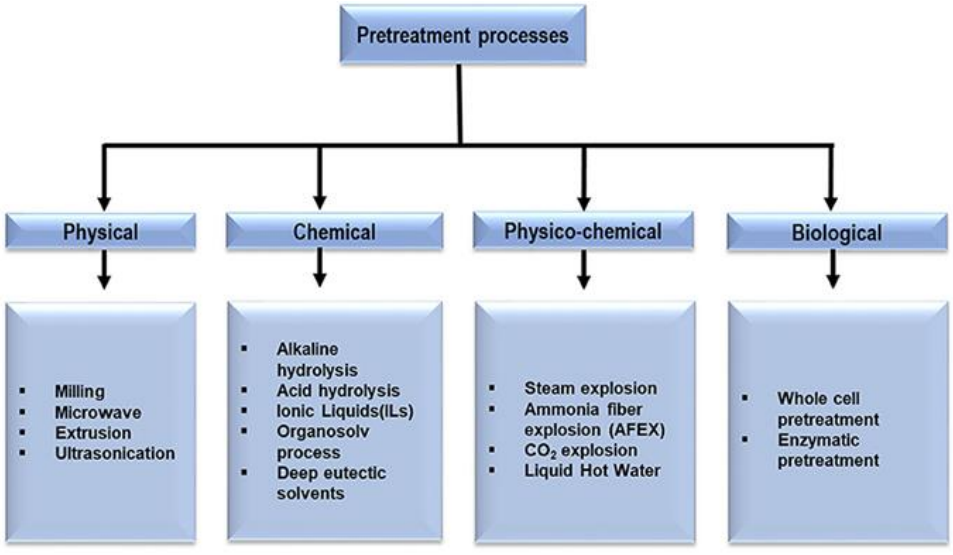

Figure 1.2: Different pretreatments methods

According to Dahmen et al. (2019), the global annual production of lignocellulosic biomass amounted to 181.5 billion tonnes. Traditionally lignocellulosic biomass is commonly disposed of in landfills. However, this practise is hindered by significant limitations such as high

transportation costs and challenges related to incineration due to the biomass's high-water content and low calorific value. The utilisation of agricultural and forest leftovers as animal feed has also been explored (Liguori et al., 2013). Hence, rather than engaging in trash disposal and incineration practises, redirecting the garbage towards comprehensive and creative environmentally-friendly methods will enhance its worth and contribute to the conservation of natural ecosystem resources. For instance, the valorisation of lignocellulosic biomass could result in the release of various reducing sugar such as glucose, xylose which are subsequently used for the production of the highly value-added products such as second-generation biofuel, organic acids, and phenols as summarized (Table 1.4).

Table 1.3: Valorisation of lignocellulosic biomass using different pretreatment techniques for value-added products

Substrates	**Conventional type**	**Pretreatment method**	**Product**	**Reference**
Bamboo	Chemical	Modified alkaline peroxide	Bioethanol	Huang et al. (2020a)
Bamboo	Chemical	Zeolite in the presence of 0.25 M Nao	Clavulanic acid	Jeong et al. (2018)
Buckwheat hull	Physicochemical	Thermo-alkaline	Biomethane	Mirko et al. (2021)
Camphorwood saw dust	Physicochemical	Ball milling with carbon-di-oxide catalyzed hydrothermal treatment	Glucose	Yuan et al. (2016)
Corn stalk	Physicochemical	Compressed hot water	Bioethanol	Adekunle et al. (2020)
Paddy straw	Physicochemical	Glycerol thermal	Reducing sugar	Gabhane et al. (2020)
Rapeseed straw	Chemical	1% sulfuric acid	Bioethanol and succinic acid	Kuglarz et al. (2018)
Rice husk	Chemical	Acidic ionic liquid	Levulinic acid	Khan et al. (2018)
Rice straw	Chemical	Acidic ionic liquid (1-(carboxymethyl) pyridinium chloride)	Bioethanol	Abdolmaleki et al. (2021)
Rice straw	Chemical	NaOH	Biobutanol	Valles et al. (2021)
Softwood pine	Chemical	Alkali (NaOH and Na_2CO_3)	Glucose	Bay et al. (2020)
Sugarcane bagasse	Chemical	NaOH-catalyzed organosolv	Glucose	Zhang et al. (2021a)

In addition, lignocellulosic biomass such as corn cob, rice straw, sugarcane bagasse, rice bran, orange peel, banana peel, paddy straw, and wheat bran, have been explored as carbon source for the production of different enzymes such as amylase, cellulase, pectinase, xylanase, β-xylosidase by different microorganisms viz., bacteria, fungus, and yeast (Amobonye et al., 2021, Jin et al., 2020, Oke et al., 2017, Srishti et al., 2022, Ticona et al., 2021). Amongst these biomass, corn cobs and sugarcane bagasse are among the largest agricultural waste in South Africa, amounting to about 16,296,301 tons of cob and 6, 301, 11 tons of sugarcane bagasse respectively (Ogbu and Okechukwu, 2023).

In Addition, Africa has a wide variety of indigenous agricultural crops generating a significant amount of biomass as a waste which could be used as a source of polysaccharides for value-added products such as ethanol, biocatalyst oligosaccharides etc, however this agricultural crops have not been exploited. Bambara biomass is one of the few examples of the unexploited biomass especially as sole of carbon source for the production of industrial biocatalyst respectively (Arumugam et al., 2019). However, amongst all these lignocellulosic biomass, wheat bran has been reported to profoundly affect the production of different carbohydrase enzyme in fungi, as well as in bacteria. For instance, xylanase production level of 474 U/mL and 1500 U/mL were observed from *A. terreus* (Bakri et al., 2020) and *Bacillus* sp. TC-DT 13 (Rodrigues et al., 2020) respectively with wheat bran as carbon source. While cellulase levels of 20 U/mL was observed in *Cotyledon pannosa* respectively (Sharma et al., 2016). Moreover, wheat bran also supported higher production level of BGL (802 U/mL) from *Aspergillus versicolor* (Huang et al., 2021), as well as endoglucanase (55.26 IU/gds) and FPase (4.64 IU/gds) from *A. niger* NFCCl 43113 (Kumar et al., 2018).

1.4 Recombinant β-glucosidase production

The conventional production of enzymes, including BGLs, using wild-type organisms comes with different challenges as whole fungal cell fermentation produces a wide variety of enzymes along with other metabolites. One of the major challenges is the purification of the enzyme of interest from the huge enzyme mixture, a process that is both laborious and expensive (Ramos et al., 2022). This challenge has since been circumvented by the emergence of recombinant DNA technology, which allows for the specific expression of targeted proteins, including enzymes, in copious amounts in both heterologous and homologous host systems. However, the recombinant expression of microbial BGLs has mostly been carried out via heterologous hosts. Although cloning and expression of these genes may be challenging, in the long run, the ease in purification of the heterologous enzymes results in a significant reduction in enzyme

production cost. According to Garvey et al. (2013), the improvement of recombinant expression of cellulolytic enzymes, including BGLs has the capability to increase the economic feasibility of the of biofuel industry by widening the scope of biomass utilization.

Hence, many recent studies have demonstrated the production of microbial BGLs using the recombinant approach (Changming et al., 2023, Sun et al., 2022, Volkov et al., 2020). It was observed that these recombinant expressions have been carried out in bacterial (mainly *Escherichia coli*), yeast (mainly *Komatogataella phaffii*), as well as in a few fungal host systems (such as *Aspergillus* and *Trichoderma* spp.). However, while the expression of the prokaryotic BGLs gene was mainly done using *E. coli* BL21 (Chen et al., 2017b, Almeida et al., 2020, Yang et al., 2022), eukaryotic BGLs were more commonly cloned in *P. pastoris* (Gutierrez-Gutierrez et al., 2023, Karnaouri et al., 2013, Li et al., 2020b). As expected, increased production yields were observed relative to the native organism (Treebupachatsakul et al., 2015, Méndez-Líter et al., 2017). In some cases, the biochemical properties of the recombinant enzymes were observed to vary from that of the wild-type enzymes. For instance, the molecular mass of the recombinant BGL whose gene was sourced from *Phanerochaete chrysosporium* and expressed in *K. phaffi* was 133 kDa compared to 116 kDa of the natural enzyme, although this had no effect on the catalytic capabilities of both enzymes (Kawai et al., 2003). However, the significant difference between the catalytic efficiency of a BGL gene expressed in two closely related *Komatogataella* host systems, *viz*., *K . phaffi* Y-11430 (Mut+ and His+), was recently demonstrated (Changming et al., 2023). A summary of recent works on the cloning and expression of microbial β-glucosidases is presented in (Table 1.5).

Table 1.4: Recombinant microbial β-glucosidases

Source organism	Host system	Vectors	Inducer	Reference
Actinosynnema mirum	*Escherichia coli* BL21(DE3)	pGEX 4T-1	Isopropyl β- d-1-thiogalactopyranoside (IPTG)	Cui et al. (2013)
Anoxybacillus thermarum	*E. coli* BL21	pET29 (+)	IPTG	Almeida et al. (2020)
Aspergillus chevalieri	*A. oryzae* NBRC 100959	pUNA	Maltose	Senba et al. (2023)
Aspergillus aculeatus	*Trichoderma reesei*	NR	Avicel	Treebupachatsakul et al. (2015)
Aspergillus niger Nip35 c	*S. cerevisiae* CEN.PK1023A	CPOT plasmid	NR	Tang et al. (2013)
Bacillus altitudinis JYY-02	*E. coli BL21* (DE3)	pET-15b	IPTG	Yang et al. (2022)
Bacillus licheniformis CGMCC 2876	*E. coli BL21* (DE3)	pEASY-E1	IPTG	Chen et al. (2017b)
Brettanomyces anomalus	*E. coli BL21* (DE3)	pET28-YV396-His6	IPTG	Almeida et al. (2020)
Coptotermes formosanus	*P. pastoris KM71* (his4)	pPIC9	Methanol	Gutierrez-Gutierrez et al. (2023)
Jeotgalibacillus malaysiensis	*E. coli BL21* (DE3)	pET28a	IPTG	Liew et al. (2018b)
Levilactobacillus brevis TMW 1.2112	*E. coli* pBAD/Myc-His A	pBAD vector	L-arabinose	Bockwoldt and Ehrmann (2022)
Microbacterium esteraromaticum	*E. coli BL21* (DE3)	pMAL-c5X	IPTG	Quan et al. (2012)
Microbulbifer sp. ALW1	*E. coli BL21* (DE3)	pET-28a	IPTG	Jiang et al. (2021)
Myceliophthora thermophila	*Pichia pastoris* X-33	pPICZαC	Methanol	Karnaouri et al. (2013)
Paenibacillus mucilaginosus	*E. coli*	pGEX 4T-1	IPTG	Siddiqi et al. (2017)
Paenibacillus mucilaginosus	*Corynebacterium glutamicum*,	pCES208	Glucose	Siddiqi et al. (2017)
Paenibacillus mucilaginosus	*Saccharomyces cerevisiae*	pYES2.1	Galactose	Siddiqi et al. (2017)

Paenobacillus mucilaginosus	*Lactococcus lactis*	pNZ8148	Nisin-inducible	Siddiqi et al. (2017)
Pectobacterium carotovorum	*E. coli* DH5α	pTAC-MATTAG®−2	IPTG	Ibrahim et al. (2023)
Penicillium verruculosum	*Penicillium canescens* RN3-11-7	pXEG v	XylA gene promoter	Volkov et al. (2020)
Penicillium oxalicum 16	*P. pastoris* GS115	pGAPZαA	Methanol	Li et al. (2020b)
Pseudonocardia sp. 1536	*E. coli BL21*(DE3)	pGEX 4T-1GST	IPTG	Du et al. (2014)
Pyrococcus furiosus	*Pichia pastoris* X-33	pGAPZaA	Methanol	Li et al. (2013)
Saccharomycopsis fibuligera	*S. cerevisiae CEN.PK1023A*	CPOT plasmid		Tang et al. (2013)
Talaromyces amestolkiae	*P. pastoris* KM71	DH5α	Methanol	Méndez-Líter et al. (2017)
Thermotoga petrophila	*E. coli BL21*(DE3)	NA	T7 promoter	Ferreira et al. (2018)
Trametes trogii	*E. coli strain Rosetta* (DE3)	pET-28b-TtBgl3	IPTG	Qu et al. (2022)
Trichoderma harzianum	*E. coli Rosetta strain*	pET28a(+)	IPTG	Santos et al. (2016)
Trichoderma reesei QM6a	*E. coli* DH5α	pET-28a	IPTG	Vázquez-Ortega et al. (2022)
Trichoderma reesei QM 9414	*S.cerevisiae* CEN.PK1023A	CPOT plasmid		Tang et al. (2013)

*NR: Not recorded

1.5 Improvement of β-glucosidases via protein engineering

Protein engineering has become one of the effective approaches for enhancing enzyme activities and improving their stability as well as other functional properties. This approach alters the critical features of enzymes (as well as other important proteins) and bestows on them the desired functions via the modification of existing genes or the introduction of novel genes (Li et al., 2020a). Specifically, protein engineering has been found effective in enhancing the catalytic efficiency of several microbial BGLs, thus boosting their potential applicability and feasibility in various bioprocesses (Arnthong et al., 2022, Kao et al., 2021, Li et al., 2023). Although there are currently various strategies employed in protein engineering, however, the most used strategies with respect to our enzyme of interest include directed evolution, fusion protein synthesis, and rational design.

1.5.1 Directed evolution of β-glucosidases

Directed evolution emulates the natural evolution process of proteins in the laboratory at an expedited rate using different gene diversification methods such as focused mutagenesis, homologous recombination, and random mutagenesis (Qu et al., 2020). This strategy involves the selection of a native enzyme, modification of the enzyme's gene sequence, expression of the modified enzyme, and subsequent functional screening until an acceptable level of enzymatic action is achieved (Chowdhury and Maranas, 2020). Directed evolution has been demonstrated in many studies to increase the catalytic efficiency, saccharification potential, transglycosylation activity as well as the thermo- and pH stability of various BGLs (Kao et al., 2021, Yin et al., 2019, Yoav et al., 2019). For example, the thermostability of a BGL from *Paenibacillus polymyxa,* which was originally inactivated at temperatures above 50°C, was improved via error-prone PCR amplification, to attain a half-life of 12 min at 65°C, with no alteration in its kinetic parameters (González-Blasco et al., 2000). On the other hand, the same approach was utilized in increasing the adaptation of a highly thermophilic BGL from *Pyrococcus furiosus* (Lebbink et al., 2000) and *Caldicellulosiruptor saccharolyticus* (Lenz et al., 2020) to cold temperature catalysis. More recently, directed evolution was employed in increasing the catalytic efficiency and tolerance to substrate inhibition in *Chaetomella raphigera* BGL (Kao et al., 2021), while it was also instrumental in increasing the catalytic efficiency and ethanol tolerance of *Penicillium oxalicum* 16 BGL (Huang et al., 2020b). It was posited that these improvements were made possible by the replacement of key amino acid residues, formation of extra salt bridges, stabilization of the hydrophobic core, as well as the

stabilization of the quaternary structure of the proteins (González-Blasco et al., 2000, Yin et al., 2019). However, the efficiency of obtaining active mutants could still be increased via diversity generation and high-throughput screening methods, which have been identified as key areas of improvement. In this regard, the generation of smart libraries via targeted mutagenesis guided by structure or sequence information as well as the incorporation of machine learning into the directed evolution workflow, have been identified as likely routes to follow (Wu et al., 2019).

1.5.2 Fusion protein synthesis of β-glucosidases

Fusion protein synthesis is the integration of two or more protein domains to enhance the individual bioactivities of the initial proteins or to generate new functional combinations. This is achieved by the joining of different genes that code for separate proteins, followed by transcription and translation as a single protein unit (Yu et al., 2015). This protein engineering approach has been used to enhance the functionality of enzymes by synergistically combining desirable features of different enzymes in a single enzyme. This has resulted in easier protein purification, integration of sequential reactions, cofactor regeneration, and stronger substrate-enzyme binding (Hirano et al., 2019, Monterrey et al., 2022). Being a terminal accessory enzyme, BGL has been fused with key biomass-degrading enzymes to enhance the conversion of lignocellulosic biomass to monomeric sugars. In this regard, a bifunctional chimeric protein with increased thermostability was synthesised from *Paenibacillus* sp. MTCC 5639 BGL and endoglucanase. It was observed that the activity of the fusion protein resulted in a 100% increase in product generation when compared to the physical mixture of the two enzymes (Adlakha et al., 2012). In another study, *Caldicellulosiruptor saccharolyticus* genes for BGL, exoglucanase and the carbohydrate-binding modules were fused to synthesise bifunctional cellulases with 2-3 folds increased saccharification potential (Xia et al., 2019). The construction of a multi-functional cellulase/xylanase/BGL fusion enzyme was achieved using the BGL gene from *Clostridium cellulovorans* and the gene for a bifunctional cellulase/xylanase from *C. thermocellum* (Chen et al., 2019a). Similarly, a tri-functional enzyme with simultaneous β-glucosidase activity, endoglucanase activity, and exoglucanase was synthesised and expressed in *Saccharomyces cerevisiae* (Liu et al., 2018) whereas β-glucosidase enzyme with β-xylosidase and α-arabinosidase activity from *Dictyoglomus turgidum* was cloned and expressed in *E. coli* (Tong et al., 2021).

1.5.3 Rational design of β-glucosidases

The rational design of enzymes leverages the relationship between the structure and function of the protein to predict potential mutants with enhanced properties, as well as to introduce mutations via site-directed mutagenesis. This protein engineering strategy has since been used to improve enzyme activity, stability, substrate-specificity, and enantioselectivity through the introduction of disulfide bonds, the addition of surface salt bridges, rigidifying of flexibility sites, etc. (Song et al., 2023). Various studies, including some recent ones, have demonstrated the effectiveness of rational design in improving the enzyme activity, thermostability, and glucose tolerance of microbial BGL. For instance, the rational design of a BGL isolated from a soil metagenome produced three mutations *viz.*, A397R, L188A, and A262S, and it was observed that A397R was responsible for a significant increase in cellobiose activity while the other two mutations were responsible for ~100% increase in glucose tolerance (Liu et al., 2019b). More recently, hydrogen-bonding networks and hydrophobic stacking interaction in the active site were amended by targeting the Met36, Phe66, and Glu168 residues for mutagenesis, resulting in enhanced catalytic efficiency and substrate affinity of *Talaromyces leycettanus* BGL (Xia et al., 2021). The specificity of BGLs from *Thermus nonproteolyticus* and *Halothermothrix orenii* was enhanced to act on thioglycosidic substrates by substitution of hydrophobic residues in both enzymes by arginine, with no loss in enzyme turnover (Almulhim et al., 2020).

The engineering of microbial BGLs has also been achieved via semi-rational design, a modified approach which combines the merits of rational design with that of directed evolution (Li et al., 2023, Zhou et al., 2023b). Semi-rational design, like rational design, is based on the information from the protein's structure, however, it incorporates the flexibility, iteration, and the elements of trial and error of directed evolution (Ouyang et al., 2023). In the study by

Li et al. (2023), the activity, glucose tolerance, and thermostability of BGL (isolated from a mountain basin metagenome) were enhanced by semi-rational design; it was observed that the catalytic efficiency and half-life of the designed enzyme were ~ 600% and 2000% of the wild type while the glucose tolerance was 200 mM higher than the wild type. In another study, the site-directed mutagenesis of two amino acids acting as gatekeepers in *Trichoderma harzianum* BGL was recoded to change the active-site accessibility and enhance the glucose tolerance of the enzyme (Santos et al., 2019).

1.6 Immobilisation of microbial β-glucosidases

Many microbial BGLs have been observed to be unstable in extreme conditions such as high pH and temperature, thus limiting their enzymatic capabilities and, consequently their industrial applications. Thus, immobilisation is one of the major techniques that have been devised to circumvent this limitation. Generally, this process improves enzyme stability and facilitates enzyme reusability, consequently decreasing the economic cost of enzymes (Alnadari et al., 2020). BGLs from different microbial sources have been immobilised using different techniques such as chemical methods (covalent bonding and cross-linking) and physical methods (adsorption, entrapment and encapsulation) (Fig. 1.3.). Furthermore, the immobilisation of microbial BGLs has been carried out using various supporting matrices including alginate, cellulose, chitosan, gelatine, and various metallic nanoparticles (Table 1.6.). For instance, Chamoli et al. (2020), immobilised BGL from *B. subtilis* using a covalent bonding technique; subsequently, a significant improvement was observed in the pH and thermostability of the immobilised enzyme. The enzyme was recorded to retain ~70% of the initial activity after 1h incubation at 70°C, and ~85% of the initial activity after 10 operation cycles (Chamoli et al., 2020). Covalent techniques were also employed in the immobilisation of *A. niger* BGL using silica gel and glutaraldehyde as supports, with the immobilised enzyme retaining 80% activity after twenty reuse cycles during cellobiose saccharification (Jung et al., 2012).

On the other side, the crosslinking technique was also employed for BGL immobilisation. For instance, Phadungcharoen et al. (2019) immobilised BGL from *A. niger* using crosslinking with chitosan. BGL from the same source was also immobilised with chitosan microspheres, and the immobilised enzyme was successfully used for the hydrolysis of isoflavones and the production of resveratrol (Zhang et al., 2014). The physical methods of immobilisation such as adsorption using different support such as silica gel, mineral salt, and metal oxides have been noted to be cost-effective and more environmentally-friendly due to the limited steps required and the minimal amount of chemicals (de Andrades et al., 2019). On the contrary, Hu et al. (2018) studied the immobilisation of *Thermoascus aurantiacus* BGLs on amorphous cellulose using adsorption, aiming to hydrolyse soybean isoflavone glycosides into their aglycones such as daidzin and genistin. In addition, a commercial BGL from *A. niger* was immobilised by Fernández-Pacheco et al. (2021) using the same technique with sodium alginate as support and it was found to be efficient in improving wine aroma by releasing volatile compounds such as nerol and geraniol compared to the free enzymes.

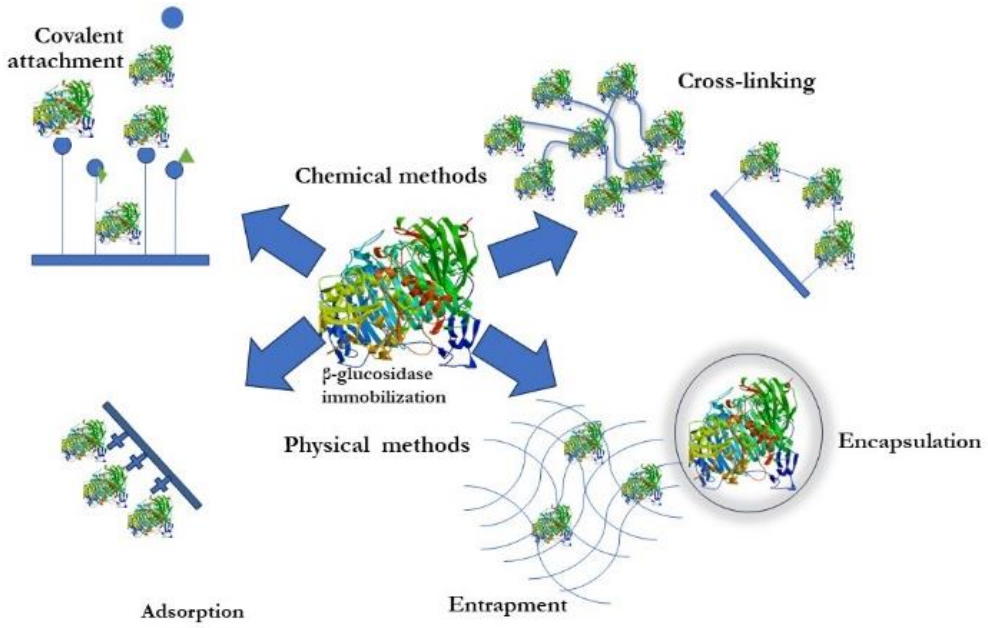

Figure 1.3: Methods of microbial β-glucosidase immobilisation

Table 1.5: Immobilisation of microbial β-glucosidase using different techniques

Source	Technique	Support	Operating stability	Reference
Aspergillus aculeatus	Covalent	Chitosan	3 cycles no <100 and 73%	de Oliveira et al. (2020)
Aspergillus awamori	Crosslinking	Gelatin	10 cycles > 80%	Nishida et al. (2018)
Aspergillus fumigatus	Covalent	Sepabeads, and relizyme™	5 cycles > 50%	Yepes et al. (2022)
Aspergillus niger	Adsorption crosslinking	Aminated MANAE-agarose	12 cycles > 66%	Rodrigues et al. (2021)
Aspergillus niger	Covalent	Magnetic nanoparticles	16 cycles = 50%	Zhou et al. (2013)
Aspergillus niger	Covalent	Sponge	8 cycles = 51%	Ahmed et al. (2013)
Aspergillus niger	Covalent coupling	Anosized silicates	5 cycles = 81%	de Andrades et al. (2019)
Aspergillus niger	Covalent	Magnetic nanoparticles	10 cycles = 60%	Park et al. (2018)
Aspergillus niger	Covalent	K-carrageenan hybrid	12 cycles > 75%	Tan and Lee (2015)
Aspergillus niger wu-16	Co-Precipitation	1,4-benzenedicarboxylicacid	8 cycles = 55%	Cao et al. (2023)
Aspergillus versicolor	Entrapment	Magnetic mno2	8 cycles > 60%	Huang et al. (2021)
Bacillus subtilis	Crosslinking	Glutaraldehyde-activated chitosan beads	5 cycles > 60%	Wang et al. (2019)
Bacillus subtilis	Covalent	Sio2 nanoparticles	10 cycles = 70%	Tan and Lee (2015)
Bacillus subtilis PS-5CM-UM3	Covalent	Sio2 nanoparticle	10 cycles = 70%	Agrawal et al. (2016)
Exiguobacterium sp	Chitosan Carbon	Crosslinking	40 cycles > 80%	Chang et al. (2013)
Humicola. insolens,	Covalent	Ferromagnetic nanoparticles	5 cycles = 80%	Carli et al. (2019)
Malbranchea pulchella	Ionicadsorption/Affinity adsorption	MANAE-agarose/cona-sepharose	20 cycles= 80%	Monteiro et al. (2019)
Phanerochaete chrysosporium	Covalent	Magnetic graphene oxide	10 cycles > 80%	Paz-Cedeno et al. (2021)
Thermatoga maritima	Covalent	Magnetic nanoparticles (mnps)	10 cycles = 66%	Alnadari et al. (2020)
Thermoascus aurantiacus	Adsorption	Polyacrylamide cryogel	7 cycles = 100%	Mól et al. (2023b)

Thermoascus aurantiacus IFO9748	Adsorption	Amorphous cellulose	30 cycles > 90%	Hu et al. (2018)
Trichoderma reesei	Adsorption/ covalent	Polyacrylicresin/ glyoxyl agarose	6 cycles >50%	Borges et al. (2014)
Zobellella denitrificans VIT SB117	Entrapment	Alginate beads	11 cycles = 50%	Mahapatra and Manian (2020)

1.7 Structural classification of β-glucosidase

β-glucosidases (BGLs) are glycosyl hydrolase that are classified based on their substrate specificity or structural features. Based on substrate specificity, BGLs can be categorised into three groups: aryl-BGLs, which exclusively hydrolyse aryl-β-glucoside bonds; cellobiases, which specifically hydrolyse cellobiose; and BGLs with broad substrate specificity, capable of hydrolysing a diverse range of substrates with various types of bonds, such as β (1→4), β (1→3), β (1→6), and α (1→6) bonds (Jiang et al., 2021). Regarding the structural characteristics of BGLs, they primarily fall into two families: glycoside hydrolase (GH) family 1 and GH family 3. GH family 1 BGLs exhibit a typical (α/β)8 TIM-barrel structure with a pocket-like catalytic channel (Zhang et al., 2022), as depicted in Fig 1.4. (a,b). On the other hand, GH family 3 BGLs possess a more intricate structure, which includes the (β/α)8 TIM-barrel fold, the (α/β)6 sandwich domain, and the FnIII domain of unknown function (Wang et al., 2020), as illustrated in Fig. 1.4. (c,d). Typically, β-glucosidases (BGLs) belonging to the GH1 family are commonly observed in archaea, plants, and animals, while GH3 BGLs are mostly from bacteria, fungi, and yeasts (Ahmed et al., 2017).

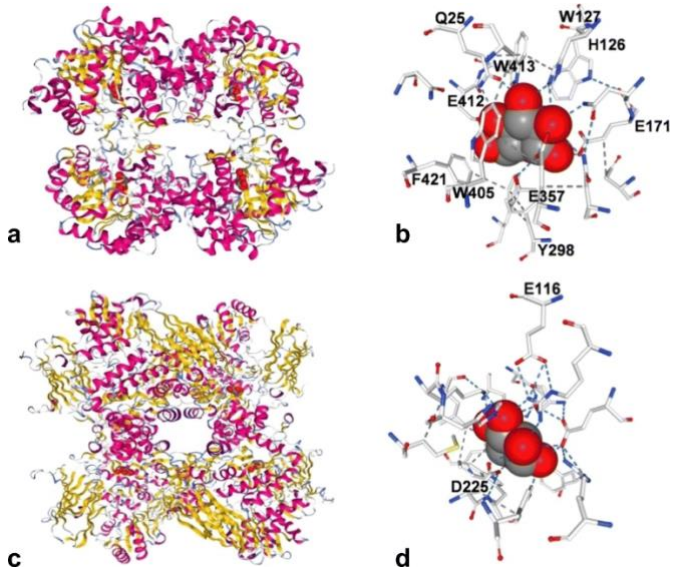

Figure 1.4: The overall structure of and active site of tetrameric β-glucosidase enzymes belonging to the GH1 family (a), (b) and GH3 family (c),(d)

1.7.1 Functional analysis of β-glucosidases

Understanding the compartmentation and localization of proteins and enzyme is important for comprehending their biological functions and the reactions they catalyse. In addition, it is

believed that information in the compartmentation and localization is important for understanding their enzymatic pathway and regulation (Ward et al., 2023, Wei, 2019). Therefore, numerous biophysical techniques have been developed to determine the secondary and tertiary structures. This includes Circular dichroism (CD), X-ray crystallography, fluorescence spectroscopy (FS), nuclear magnetic resonance cryogenic electron microscopy, etc.

X-ray crystallography is a well-known method that has played an important role in determining the molecular mechanism of many biological processes. For instance, over 163,949 structures have been deposited in protein data bank (PDB) whereby x-ray crystallography contributed to the characterisation of over 80% of them respectively (Ojoawo, 2020). This technique has been observed to be accurate and does not require a large number of the sample as one single crystal structure is more than enough for structure determination, however, there is one major limitation of this method which is obtaining high- quality crystals especially when the protein to be analysed is large and contain structural flexible regions such as loops and intrinsically disorder regions, etc., (Feng et al., 2011).

The structures of glycosyl hydrolase including BGL have been characterized using X-ray crystallography to improve their properties such as glucose tolerance and thermostability. For instance, metagenomic BGL structure was characterized by X-ray crystallography in order to determine the amino acids that can be manipulated through mutation to improve thermostability. The metabolic structure obtained contained TIM-barrel fold $(\beta/\alpha)_8$ and several external α-helices and β-barrels (Fig 1.5.A). In addition, amino acids responsible for thermostability were reported to be Glu356, Glu170, and Glu410 as shown in Fig 1.5 (B) respectively (Matsuzawa et al., 2017). In addition, X-ray crystallography, and molecular dynamic simulation was also employed to evaluate the structural basis responsible for BGL from *Exiguobacterium antarcticum* B7 to thrive in low temperatures. It was discovered that *Exiguobacterium antarcticum* B7 BGL possess a tetrameric arrangement which is kind of unique in GH1 family due to their subunits that assemble in different way from those reported within GH1 family in the PDB (Fig. 1.6) (Zanphorlin et al., 2016).

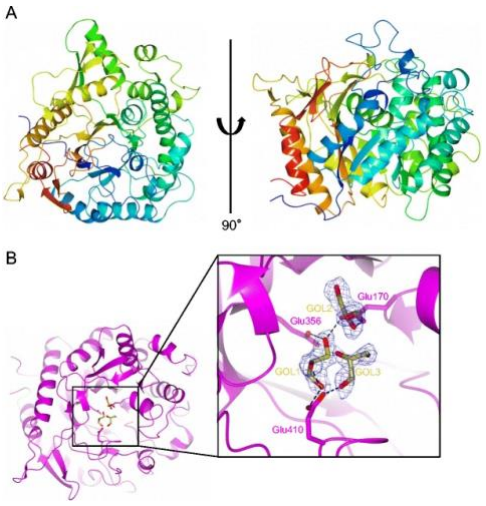

Figure 1.5: (A), The crystal structure of MeBglD2 is shown as a multi-colour ribbon model. (B) The active site of the enzyme with three glycerol molecules is shown as a magenta-coloured ribbon model with a black box

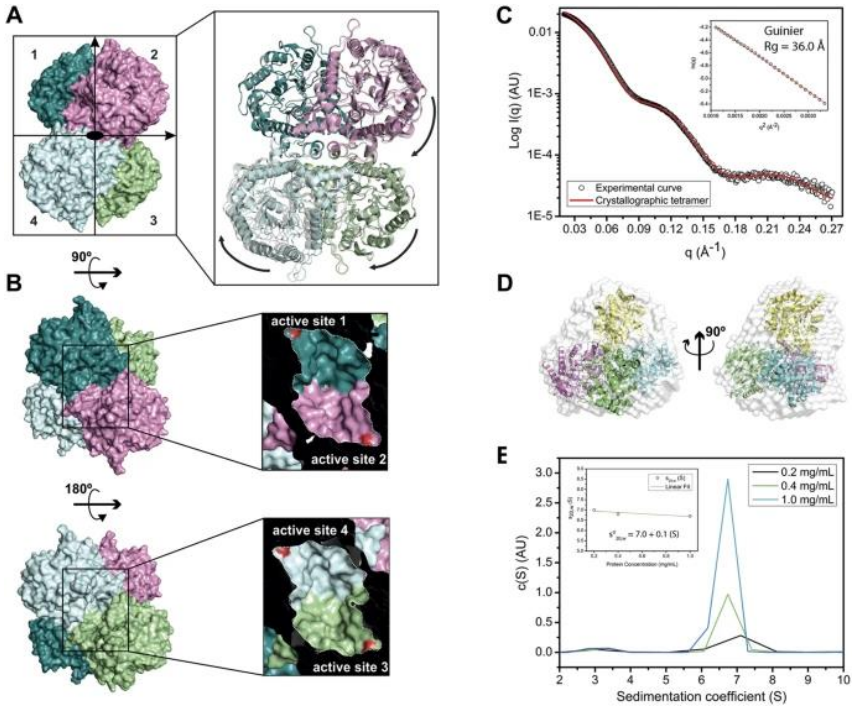

Figure 1.6: A. The crystal structures of EaBglA reveal a tetrameric arrangement with 222 symmetry. The two independent tetramers observed in the P21 and C2221 crystals (multicolour and gray) are in slightly different conformations, suggesting inter-subunits motions (arrows). (B) Two pairs of interfacing active sites (1–2 and 3–4) locate at opposite faces of the tetramer. SAXS profile (C) and ab-initio model (D) agree with the theoretical curve and high-resolution structure of the tetramer. (E) AUC assays show the presence of tetramers in solution, even at low protein concentrations

1.7.2 Nuclear magnetic resonance (NMR) spectroscopy

Based on the numbers of structures deposited in the PDB in recent years, NMR has become the second most approach used for characterizing various biological molecules after X-ray crystallography (Ojoawo, 2020). NMR characterise the structure by using magnetic spin properties of atomic nuclei. NMR is considered inexpensive and less technically demanding as opposed to X-ray crystallography because NMR does not require a crystalised sample (Puthenveetil and Vinogradova, 2019). In addition, NMR signals can only be observed for nuclei with net spin, and subsequently in order for abundance, isotopes that are commonly used in biomolecular NMR are ^{1}H, ^{31}P, ^{13}C, ^{15}N, and ^{2}H, respectively (Marion, 2013). In order to obtain a high-quality spectra, a protein sample must be isotopically labelled either uniformly or selectively with ^{13}C, and ^{15}N subsequently (Ojoawo, 2020). Recently, NMR has been employed to study the structure of recombinant BGL from the lactic acid bacteria *Oenococcus oeni*. The results obtained from this study showed that chemical shift values of hydrogen in recombinant BGL range between δ0.03 ppm to δ10.25 ppm, suggesting that these hydrogen could be divided into three categories due to their different chemical shift as depicted in Fig 1.7. The signal relating to the amino acids residue of CH3, CH2, and CH were identified as chemical shift values ranges within δ0.03 ppm to δ1.8 ppm. The chemical shift values ranges between δ3.3 ppm and δ4.0 ppm were thought to be associated with the R-O-CH3 amino acid residue. While the residual hydrogen was concentrated in an area typical of aromatic hydrogen, with chemical shift values between δ6.75 ppm and δ7.75 ppm (Zhang et al., 2021b).

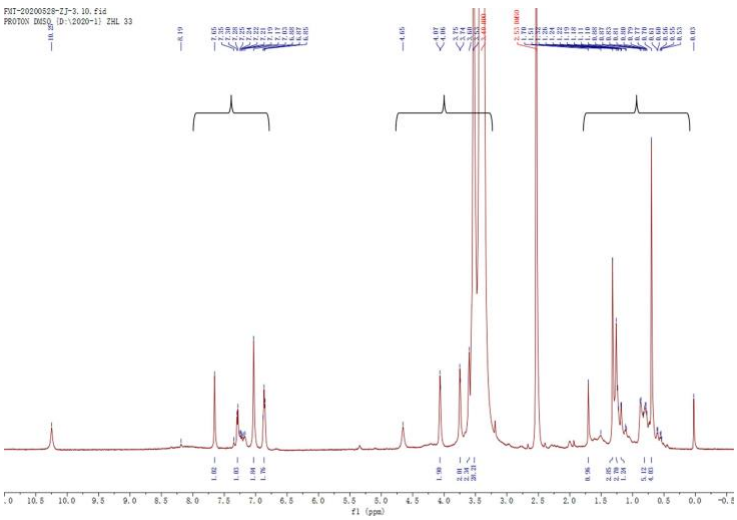

Figure 1.7: 1H NMR spectrum of *Oenococcus oeni* BGL (Ojoawo, 2020)

Moreover, there is another biophysical tool which has gained popularity over X-ray crystallography, known as 3D cryo-electron microscopy (Cryo -EM) which does not require a crystalised sample. This structural biology technique directly visualized the biological samples using transmission electron microscopy (TEM), which eventually generate 2D images corresponding to a projection of the structure in the direction of the electron path (Fig. 1.8) (Callaway, 2020).

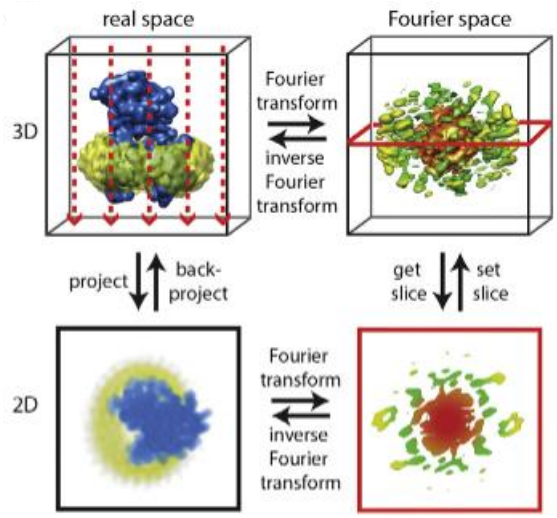

Figure 1.8: The projection-slicorem states that the 2D projection of a 3D object in real space (left column)is equivalent to taking a central 2D slice out of the 3D Fourier transform of that object (right column). The real-space projection direction (left, dashed red arrows) is perpendicular to the slice (right, red frame) (Callaway, 2020).

However, due to the complexity of biological system, these biophysical techniques are highly technical and expensive. In addition, one method is usually not enough to provide the detailed structural and dynamic properties of that analysed molecules, therefore the focus has been on to develop affordable, robust, and effective technique to improve and accelerate the protein structural determination process (Cerofolini et al., 2019, Ojoawo, 2020). In this regard, computational approaches, via systems and algorithms have been observed as alternatives for protein structural determination in recent decades. These methods have been noted to be less technical, inexpensive, and fast, however computational approach have less accuracy compared to conventional approach because they are mainly based on predictions (Siebenmorgen and Zacharias, 2020).

Computational tools have been reported to also play a crucial role in the design of enzymes by facilitating the reduction of misfolding of residues and the identification of catalytic regions,

hence, enhancing substrate binding capabilities. For example, enzymes possess free spaces and catalytic domains that accommodate substrates for the purpose of bioconversion, hence, the utilisation of biocomputational techniques has the potential to enhance the biological activity of enzymes (Holliday et al., 2011). Various computational technique such as *in silico* analysis including molecular docking which elucidates both the structural and functional characteristics of enzymes has been developed. Furthermore, it provides valuable insights into the number of steps involved in a chemical reaction, the specific catalytic site within enzymes, the presence of amino acids in the enzyme, the availability of cofactors for enzymatic activity, and the chemical alterations that take place during the reaction (Adelusi et al., 2022, Danel et al., 2023). In addition to computation approach, another technique is Molecular dynamics simulation (MDS) which used for the determination of the binding affinity between enzymes and substrates or ligands, as well as the assessment of enzyme stability. This technique was invented to also assist in protein structural determination as well as in other field e.g. drug development (Danel et al., 2023, Hollingsworth and Dror, 2018).

Numerous BGL from different families have been characterised structurally by *in silico* analysis with the objectives of elucidating their 3D structure and as preliminary steps to their functional modifications via protein engineering. Secondary and tertiary structure predictions of various BGL have been generated using SWISS-MODEL tool, GOR-IV tool, AutoDock, AutoDock Vina, PSI -Dockvina, and PROCHECK etc, for determination of α-helix, β-sheet, turns, and loops (Albalawi et al., 2023, Purohit et al., 2023, Ramachandran et al., 2012). Recently 3D structure of BGL (MaBgl3) from *Microcystis aeruginosa* CACIAM 03 was determined using *in silico* analysis approach (Serra et al., 2023). Additionally, molecular docking between two substrates cellobiose (CBI) and β-D-glucose (BGC) obtained from PubChem was also evaluated followed by MDS respectively. The 3D structures predictions shows that *M. aeruginosa* BGL contained (β/α)8 TIM-barrel fold which is often typical of GH3 family glycosylases (Fig. 1.9).

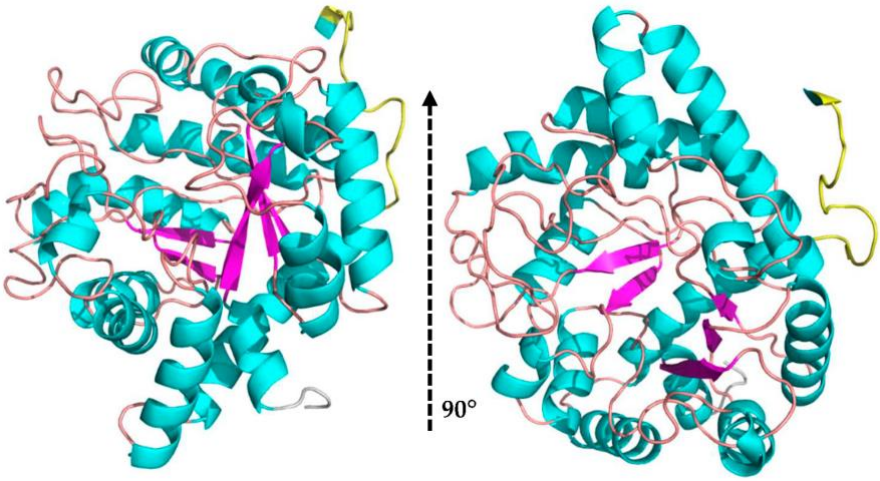

Figure 1. 9: N-terminal domain folded as a distorted TIM barrel in the MaBgl3 model (β/α)8 in a side view and upwards view (cartoon structure: α-helices in cyan, β-sheets in magenta, and loops in beige; linker loop in yellow; N-terminal end in grey colour) (Serra et al., 2023)

The molecular docking results revealed that MaBgl3 had a higher binding affinity for cellobiose compared to glucose, due to the lower binding free energy obtained between MaBgl3-CBI complex (-7.10) when compared to MaBgl3-BGC complex (-6.18). In addition, more interactions were observed between MaBgl3-CBI complex, however, both ligand formed either a hydrogen bond with MaBgl3 amino acids residues. BGC formed hydrogen interactions with Asp81, Asp270, Phe443, whereas CBI formed hydrogen bonds with Trp31, Asp81, Arg444 respectively. Asp81 was observed to be responsible for proper interaction between the MaBgl3 and these two ligand as these residues were obtained in both complex hydrogen interactions. Moreover, MDS of MaBgl3 and the chosen ligand was stable, revealed by the root mean square deviation (RMSD) observed for both MaBgl3-CBI complex (2.09 ± 0.34 Å) and MaBgl3-BGC (2.82 ± 0.38 Å), which subsequently fall under the acceptance standards.

In another study, computational approach revealed a high efficiency, thermos-stable, and glucose-tolerant BGLs from *Paenibacillus lautus* BHU3 strain through molecular modelling, docking, and simulation dynamics respectively. In this study seven BGLs were identified from the genome of *Paenibacillus lautus* BHU3, out of them one was of glycosyl hydrolase-1 family and six of them belonged to the glycosyl hydrolase-3 family. The predicted physiological properties of the identified BGLs revealed an acidophile with pI ranges between 4.7-5.5. Furthermore, a higher thermostability of the BGLs proved by higher T_m values ranged from 65.4-75.8 as well as higher aliphatic index ranged from 81.48 and 98.86. respectively (Yadav et al., 2021).

Subsequently, substrate specificity of the identified BGLs from *Paenibacillus lautus* BHU3 was predicted by molecular docking using two substrate, cellobiose and p-nitrophenyl-B-D-1,4-glucopyranosidae (pNPG). All identified BGLs showed a higher binding affinity for both substrates due to higher binding free energy obtained ranging from −6.434 to −9.054 kcal mol^{-1} for pNPG and −11.33 to −13.293 kcal mol^{-1} for cellobiose respectively, thus providing a high binding affinity of the identified enzymes for cellobiose over pNPG. Further analysis such as MD simulations and glucose tolerance was carried out using WP_096774744.1 and pNPG complex. MD simulations revealed the Glu5, Arg7, Lue68, Gly69, and Phe325 as major residues responsible for glycosidic bond hydrolysis. The WP_096774744.1- pNPG complex reached the stability within the first 1 ns and it remained stable throughout the simulations. Therefore, according to this data, computational approach has shown to be a highly promising and cost- effective technique to characterize protein based on their structures using the available information in the NCBI protein database.

1.8 Industrial application of microbial β-glucosidases

β-glucosidase from various microbes has been found useful in various biotechnological applications (Fig. 1.10). Currently, this enzyme has biotechnological potential in the production of renewable energy sources (biofuels) from lignocellulosic biomass; in the enhancement of food/ drinks aroma and flavour via the enzymatic release of aromatic compounds; as well as in the synthesis of alkyl-glucosides, oligosaccharides, and glycoconjugates which are important compounds in the pharmaceutical industry. Furthermore, BGL has also been utilized in the cosmetic industry for the synthesis of depigmenting agents, As a result, there is a growing market for this enzyme, and it is expected to continue to expand in the future.

1.8.1 β-glucosidase in bioethanol production

In recent years, there has been a growing interest in the development of biofuels, particularly bioethanol, as a viable means of mitigating the emission of greenhouse gases resulting from the combustion of fossil fuels (Sun et al., 2022). The enzymatic hydrolysis of cellulose by cellulase enzymes, including endoglucanases, exoglucanase, and β-glucosidase, has been identified as a crucial step in the saccharification stage for bioethanol production. While endoglucanases attack the internal region of cellulose, causing depolymerization of cellulose structure, exoglucanases break down β-glucan molecules by releasing cellobiose (Amobonye et al., 2021). Subsequently, cellobiose is further broken down by BGL into glucose, which is fermented by yeasts, such as *S. cerevisiae* and *K. phaffi* to produce bioethanol (Zhou et al.,

2023a). Thus, the catalytic activity of BGL is a critical stage in the cellulose breakdown process due to its role in regulating the efficiency of hydrolysis and its potential to alleviate the feed-back inhibitory effects of cellobiose on exoglucanase and endoglucanase (Zhao et al., 2022). In this regard, the supplementation of exogenous BGLs into the commercial cellulase has been noted to significantly improve reducing sugar concentration leading to more efficient biofuel production (Mól et al., 2023a).

For instance, the addition of recombinant BGL derived from *Anoxybacillus flavithermus* to a commercial cellulase, led to a 3.4-fold increase in the conversion of sugarcane bagasse to glucose, compared to the use of Celluclast alone (Liu et al., 2017b). In another study, the hydrolysis of sugarcane bagasse was found to be more efficient when commercial cellulase was used in conjunction with *Aspergillus saccharolyticus* BGL, compared to when used alone. The BGL from *Pholiota adiposa* was also demonstrated to exhibit significant catalytic efficacy towards cellobiose as it increased the efficiency of rice straw biomass saccharification by 24% (Godse et al., 2021). Various bacterial BGL have also been bioprospected for use in biomass saccharification (Sheng et al., 2016). For example, the BGL from *Ruminiclostridium thermocellum*, a thermophilic bacterium isolated from horse dung, showed immense capabilities in saccharifying corn cobs, cornstalks, poplar sawdust, and rice straw (Sheng et al., 2016). Furthermore, the thermostable *B. subtilis* RA10 BGL facilitated efficient saccharification of paddy straw into fermentable sugars, and the supplementation of commercial cellulase with this enzyme resulted in 34% increase in glucose release (Tiwari et al., 2017). Another noteworthy example is the use of BGL, derived from the marine bacteria *Alteromonas australica*, which was successfully inserted into *E. coli*; the enzyme had a significant capacity to withstand glucose and demonstrated optimal activation when exposed to a glucose concentration of 100 mM (Sun et al., 2020). However, there still remains the continued pursuit of BGLs with improved characteristics, such as glucose tolerance, wide-range pH stability, and better temperature stability for increased efficiency in biofuel production.

1.8.2 β-glucosidases in the beverage industry

The major saccharification function of BGLs and its many other side catalytic reactions, especially transglycosylation, have facilitated their utilization in the production of various beverages. Wine and juices, which are commonly produced from grapes and other fruits, are known to contain some non-volatile glycosidic complexes such as geraniol, nerol, and citronelloli (Haslbeck et al., 2018). These non-volatile glycosidic complexes are mainly

responsible for wine aroma and taste; however, their effects may be further enhanced by BGL deglycosylation leading to improved wine complexity and taste (Zhang et al., 2021c). Although the activity of endogenous plant BGL may also play some roles, however, this process is typically lengthy and time-consuming, hence, the need for supplementation with exogenous BGLs (Singh et al., 2016).

de Ovalle et al. (2018) demonstrated the activity of *Issatchenkia terricola* BGL on aroma precursors from the wine Cabernet Sauvignon, and the analysis of the released aglycones showed a significant increase in the concentration of several volatile compounds indicating the ability of the BGLs to liberate norisoprenoids and phenols from their respective precursors. However, the major limitation of BGLs in this regard is that the majority of microbial BGLs are sensitive to the severe conditions necessary for wine ageing such as low temperature, low pH, and high ethanol concentration (Gao et al., 2022). To this end, numerous studies have been conducted to identify BGLs with significant tolerance to these wine-aging conditions. Recently, a BGL from *Lactobacillus brevis* was observed to improve the aroma of strawberry wine and it was also observed to display strong acid and ethanol tolerance (Li et al., 2022).

Furthermore, BGLs from the non-*Saccharomyces* yeasts, *Meyerozyma guilliermondii* NM218 and *Hanseniaspora uvarum* BF345, were applied to wines and left to age for 50 days (Gao et al., 2022). It was observed that the enzyme increased the total terpenoids of the beverage from 65% to 70%. In addition, the two BGLs retained ~ 80% of their original activity at lower temperatures (20°C), lower pH (pH 3.0 - 4.0), and at high ethanol concentration (15%). Similarly, wines subjected to treatment with the two BGLs exhibited higher concentrations of hexanol, phenylethanol, ethyl octanoate, ethyl heptanoate, and ethyl caprate as well as better taste profile compared to wines without any enzymatic treatment or those treated with only commercial BGL (Gao et al., 2022).

BGLs have also been utilized in the removal of cyanogenic glucosides from sorghum malt during the production of African beer, with the enzyme hydrolysing the recalcitrant cyanogenic glucoside, dhurrin, to generate sufficient precursors for beer bioflavouring (Tokpohozin et al., 2016). Besides their more studied applications in the production of alcoholic beverages, BGLs have also been established to be effective in improving the quality of non-alcoholic beverages including tea (Liang et al., 2023) and juices (Godse et al., 2023, Miao and Zhong, 2023). For instance, a BGL from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China) was observed to improve the aroma, colour, and overall suitability of fermented black tea juice when added during the fermentation of the tea leaves (Liang et al., 2023). Recently

Candida. *sorbosivorans* BGL was also used to improve the aroma of fermented mango juice by generating 41 different metabolites (Miao and Zhong, 2023b). BGLs have also been noted to be utilised in the synthesis of natural sweetening and solubilizing agents such as ruboside. For example, a BGL isolated from *Chryseobacterium scophthalmum* was observed to efficiently hydrolyse stevioside to produce rubusoside with 99% yield, suggesting that the BGL could be a promising biocatalyst for the industry-scale production of rubusoside (Yan et al., 2021).

1.8.3 Therapeutic uses of β-glucosidase

The transglycosylation activity of BGLs is one of the significant characteristics which facilitate the production of alkyl- glucosides, oligosaccharides, and glucoconjugates, thus highlighting their applicability in the pharmaceutical industry (Tran et al., 2023, Xia et al., 2022). For instance, BGLs have been found useful in the production of gluco-oligosaccharides, a promising class of prebiotics that has attracted attention from scientists and the industry. For example, *A. niger* BGL was demonstrated to catalyse the transglycosylation of maltose and cellobiose to release the oligosaccharides, panose and cellotriose (Xia et al., 2022). Similarly, BGL from *Coprinopsis cinerea* catalysed the transfer of a glucosyl residue from laminarioligosaccharide to aminarioligosaccharide resulting in the production of laminarioligosaccharides, which had approximately double the antioxidant activity than the control (Kang et al., 2019). BGLs have also been found effective in the biotransformation of various bioactive plant metabolites such as diadzin, genistin, sesaminol glycosides, hesperidin, neohesperidin, naringin, narirutin, rutin, and neoponcirin, into their more potent products (Grynkiewicz, 2020, Mól et al., 2023a). The plant compounds have been linked to antioxidant, anticancer, anti-inflammatory, and estrogenic properties, however, their intestinal absorption in their original glycoside forms is limited (Nagula and Wairkar, 2019). Hence, the absorption and bioavailability of these phenolic glycosides were enhanced through BGL hydrolysis (Singhvi and Zinjarde, 2020).

The production of pharmaceutically significant glycosides such as epigallocatechin gallate was recorded to be facilitated by a glucose-tolerant BGL derived from *Talaromyces amestolkiae* by means of transglycosylation (Méndez-Líter et al., 2020). The transglycosylation capabilities of two recombinant BGLs derived from *Talaromyces amestolkiae* were also established as the two enzymes facilitated conversion rates of 13.8% and 19% for vanillyl alcohol and hydroxytyrosol respectively (Méndez-Líter et al., 2019). Both of these glycosides were subsequently discovered to exhibit superior anti-tumour effects on breast cancer cells compared to their

respective aglycones (Méndez-Líter et al., 2019). The industrial potential of microbial BGLs to augment the bioactivities of soybean metabolites (mainly by the biotransformation of inactive glycosides to bioactive isoflavones), has also been highlighted in previous studies (Delgado et al., 2019, Tran et al., 2023). Recently, the colorectal chemopreventive and immunization potential of anthocyanin extracted from Northern Thai purple rice was found to be enhanced by the biotransformative ability of BGLs from a *Lactobacillus* spp. (Sirilun et al., 2022). Hence, the aforementioned qualities suggest the possible utilisation of BGL-based transglycosylation in the production of uncommon oligosaccharides with significant therapeutic value.

1.8.4 Other applications of microbial β-glucosidases

Microbial BGL has also been found useful in other applications besides the key ones highlighted above. One of these is the utilization of BGLs in the production of non-lactose milk to cater for lactose-intolerant consumers. Typically, lactose intolerant individuals are deficient in β-D- galactosidase enzyme which breaks down lactose into glucose and galactose, consequently, individuals affected by this deficiency experience symptoms such as diarrhoea, bloating, and gastrointestinal issues when they consume milk or milk products (Kannan et al., 2023). In this regard, many BGLs have been described to possess galactosidase activities. For instance, *Bacillus* sp. D1 BGL which had additional galactosidase and transglycosidation activity was demonstrated to hydrolyse ~90% of milk lactose while also producing significant amounts of beneficial galacto-oligosaccharide (Deng et al., 2020a). More recently, a recombinant BGL from *Bacillus velezensis* hydrolysed ~ 40% of bovine milk after 4 h incubation, further highlighting BGLs as an industrial biocatalyst in the dairy industry (Liu et al., 2021). Cassava, a staple food in Africa, has been demonstrated to be detoxified by BGL as demonstrated by Murugan et al. (2012), where BGLs from *Bacillus subtilis* KM05 biotransformed the cyanogenic glycoside in the crop to the less harmful hydrogen cyanide and ammonia. Similarly, microbes with significant BGL activity, especially, *Lactobacillus plantarum* LBZ46, were isolated from cassava and were further demonstrated to be effective in the controlled fermentation of cassava dough (Bouatenin et al., 2019).

BGLs have also demonstrated potential utility in the cosmetic industry as well. For instance, *Thermotoga neapolitana* BGL was used to synthesise arbutin, a glycosidic molecule that enables skin whitening (Kannan et al., 2023). Furthermore, BGLs have also been used to convert polydatin into resveratrol, a polyphenolic phytoalexin which has been scientifically proven to have health-promoting properties such as antiproliferative, anti-angiogenic, anti-

inflammatory, antioxidant, and antimicrobial properties (Zhou et al., 2022). For example, resveratrol has gained popularity in cosmetology and dermatology due to its ability to penetrate the skin barrier and anti-ageing activity (Ratz-Łyko and Arct, 2019).

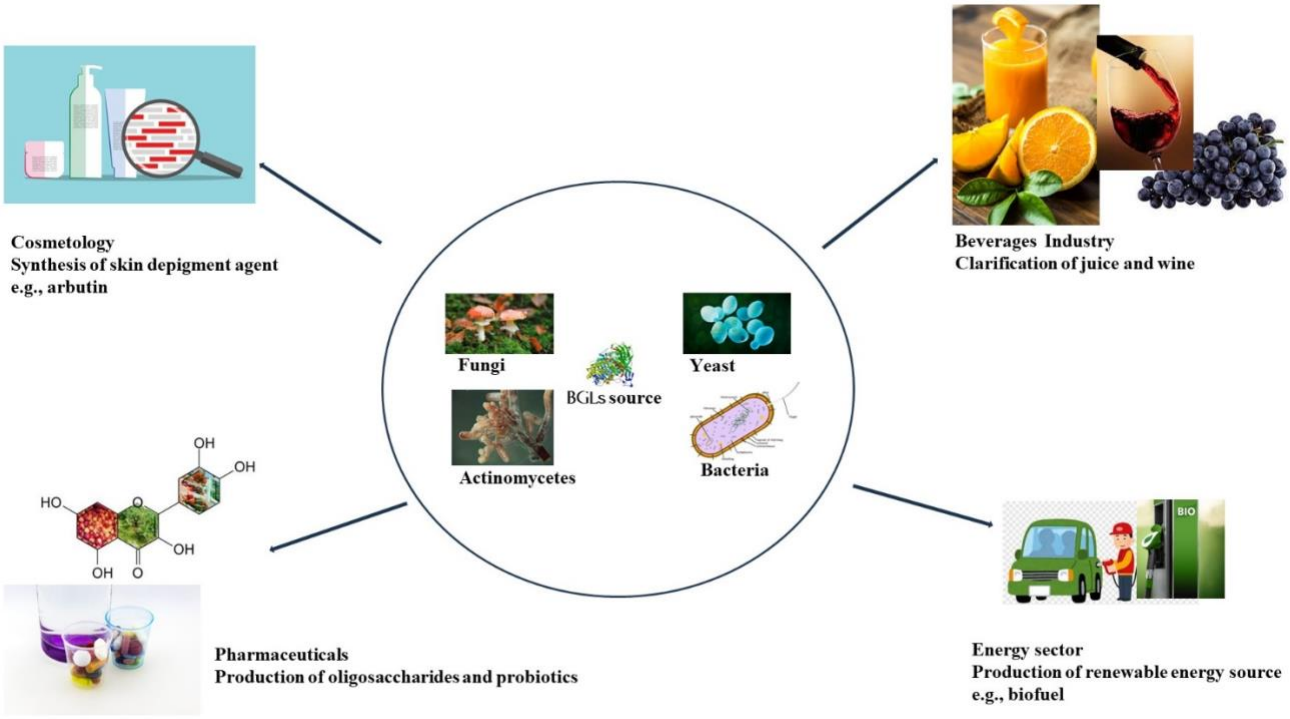

Figure 1.10: Applications of β-glucosidase in the biotechnological industries

1.9 Commercial status of microbial β-glucosidases

BGL constitutes more than one-quarter of the global agricultural enzymes market, which was valued at approximately USD 380 million in 2021 and is expected to increase to approximately USD 760.23 million by 2027 at a compound annual growth rate (CAGR) of 12.08% (360iResearch, 2021). It was observed that the huge demand is being mainly propelled by its various uses in the food industry and in the biofuel industry. For instance, BGLs have been demonstrated to breakdown lactose in milk, making the dairy product more suitable for lactose-intolerant consumers. Furthermore, they serve as a processing aid to improve the quality, flavour, and nutritional properties of other dairy products (Deng et al., 2020b). Although various companies including Megazyme, Sigma-Aldrich, Merck, and Thermoscientific manufacture the enzyme for research and analytical purposes, there are still setbacks for scaling up the production of BGL for industrial use. This is because there are complex mix of challenges such as enzyme stability and activity, high production cost, enzyme purification and recovery, process optimisation, regulatory and safety concerns, genetic engineering and strain

improvement and economic viability, and market competition- all of which needs to be effectively addressed.

However, patent information on BGL demonstrated the industrial impact of microbial BGLs in the last two decades. In this regard, a brief patent review covering the years 2000 to 2023 was performed to highlight the latest developments and successes recorded by various research groups and industries in the applicability of BGLs. The information gathered from patent databases including Canada Patent Office, European Patent Office, China Patent, and US Patent Office, were reviewed, and summarized in Table 1.7. The search revealed 26 patents and patent applications which are related to either the production or application of BGL in the industry. It was also observed that more than 50% of these patent activities were carried out in the United States. It should be noted that most of the patents are owned by companies and not by universities, with companies such as Danisco US Inc., Iogen Energy Corp, and Novozymes Inc., leading the pack.

Table 1.6: Patents related to microbial β-glucosidases

Patent no	Invention	Highlight(s)	Assignee	Reference
CA2776170C	Recombinant c1. beta-glucosidase for the production of sugars from cellulosic biomass	Expression of a recombinant BGL for the production of fermentable sugar from cellobiose	Codexis Inc	Clark et al. (2015)
CA2097180C	Saccharification of cellulose by cloning and amplification of the beta -glucosidase gene of *Trichoderma reesei*	Expression of extracellular BGL in a filamentous fungus in a recombinant host containing enhanced, deleted, or altered expression	Danisco US Inc	Fowler et al. (2007)
CN101492661B	Cloning, expression of beta-glucosidase gene, and preparation for gentian oligose	Cloning and expression of *Aspergillus niger* WX-07 BGL in *P. pastoris;* enzyme with transglycosylation activity and can convert dextrose into the gentian oligose.	Jiangnan University	Wu et al. (2011)
CN100575483C	The method for preparing heat resistant xylanase, heat-resisting xylobiase or heat-resisting BGL	Method for preparing heat-resistant xylanase, xylobiase or BGL from thermophilic *Paecilomyces varioti* using agricultural wastes as substrate.	China Agricultural University	Jiang et al. (2009)
CN109355275B	Beta-glucosidase mutant with high thermal stability and application thereof	BGL mutant with high thermal stability and application thereof; mutant enzyme with improved thermal stability and glucose tolerance, which is obtained by mutation of wild type enzyme	Sun Yat Sen University	Xiao et al. (2013)
CN101270352B	Method for preparing beta-glucosidase fixed with magnetic nano-particle	Preparation of magnetic nanoparticle fixed BGL using iron oxides via glutaric dialdehyde cross linkage.	Donghua University	Zhu et al. (2010)
CN102740868B	For producing the restructuring beta-glucosidase enzyme variant of soluble sugar from cellulose biomass	Recombinant expressed of the variant form of fungi C1 bacterial strain BGL, application of enzyme to produce fermentable sugar from biomass for eventual biofuel production.	Codexis Inc	Yang et al. (2016)
CN102220302B	Beta-glucosidase mutant, recombined expression	BGL mutant, a recombined expression plasmid and a converted engineering strain via gene site-directed mutagenesis; mutant and engineered strain with	Anhui University	Xiao et al. (2013)

	plasmid and converted engineering strain	improved glucose tolerant concentration and industrial value. Used for the hydrolysis of methyl cellulose, and fruit juice processing.		
CN101384713B	Treatment of cellulosic material and enzymes useful therein	Utilization of BGL together with other lignocellulosic enzymes for sugar hydrolysates production from cellulosic material.	Rohr Inc	Vehmaanperä et al. (2013)
CN101255449B	Clone, expression of beta-glucosidase gene, and preparation for gentian oligose	BGL used in polydatin transformation and polydatin preparation with considerable economic benefit.	Shandong University	Wu et al. (2010)
EP2599862B1	Beta-glucosidase variants with improved properties	BGL variants, nucleic acids encoding the variants, methods of use, and methods of identifying additional variants	Danisco US Inc	Bott et al. (2016)
EP2342329B1	Beta-glucosidase variants having improved activity, and uses thereof	Expression and optimisation of enzymes involved in the degradation of lignocellulosic biomass; BGL variants of *Trichoderma reesei* BGL as well as the use of these improved performance variants in the cellulose hydrolysis and biofuel production processes.	IFP Energies Nouvelles IFPEN Proteus SA	Lopes-Ferreira et al. (2016)
JP2009-190840	Protein having β-glucosidase activity and uses thereof	Novel, highly hydrophobic β-glucosidase from *Acremonium cellulolyticus*, a gene corresponding to the identified enzyme and expressed in *Trichoderma viride*	Meiji Seika Pharma Co Ltd	Yokoyama et al. (2018)
NZ561247A	Beta-glucosidases, nucleic acids encoding them and methods for making and using them	BGL with industrial use as a property modifier, e.g. as a dough-conditioning agent, in beverages, and when making paper and textile products.	Verenium Corp	Blum et al. (2010)
US9080165B2	Variants of beta-glucosidase	Nucleotide sequences encoding the BGL variants as well as vectors, host cells comprising the nucleotide sequences.	Novozymes Inc	Fidantsef et al. (2008)
US8604277B2	Polypeptides having beta-glucosidase activity and polynucleotides encoding same	Isolated polypeptides having BGL activity and isolated nucleotides encoding same; nucleic acid constructs, vectors, and host cells comprising the	Novozymes AS, Novozymes Inc	Krogh and Harris (2012)

		polynucleotides, methods of producing and using the enzyme.		
US10260058B2	Beta-glucosidase variants polynucleotides encoding same	BGL variants of a parent Family GH3A from *Aspergillus fumigatus;* polynucleotides encoding the; nucleic acid constructs, vectors, and host cells comprising the protein; and methods of using the enzyme variants.	Novozymes Inc	Wogulis et al. (2019)
US8486683B2	Beta-glucosidase enzymes	Modified BGLs from *Trichoderma reesei* BGL with kinetic parameters (comprising amino acid substitutions, genetic constructs comprising nucleotide sequences encoding for modified enzymes, methods for the production of modified enzymes from host strains and the use of the modified enzymes in cellulose hydrolysis.	Iogen Energy Corp	Scott et al. (2013)
US8071349B2	BGL4 beta-glucosidase and nucleic acids encoding the same	Novel BGL nucleic acid sequence, and the corresponding amino acid sequence, expression vectors and host cells comprising a nucleic acid sequence encoding the enzyme, recombinant enzymes, and methods for producing the same.	Danisco US Inc	Dunn-Coleman et al. (2011)
US20200056216A1	Compositions and methods related to beta-glucosidase	BGL from *Glomerella graminicola*, polynucleotides encoding enzyme, and methods of making and/or use thereof.	Danisco US Inc	Bower et al. (2020)
US10227614B2	Polypeptides having beta-glucosidase activity, beta-xylosidase activity, or beta-glucosidase and beta-xylosidase activity and polynucleotides encoding the same	Isolated polypeptides having BGL, beta-xylosidase, or BGL and beta-xylosidase activity and polynucleotides encoding the same; nucleic acid constructs, vectors, host cells comprising the proteins and methods of producing and using the polypeptides.	Novozymes AS Novozymes Inc	Morant (2019)
US9493754B2	*Aspergillus* containing beta-glucosidase, beta-glucosidases and nucleic acids encoding the same	Novel *Aspergillus saccharolyticus* strain, BGLs encoded by the strain, and the use thereof in the degradation of lignocellulosic material; host comprising the enzyme sequence and/or polynucleotides encoding same.	Clean-Vantage LLC	Teller et al. (2016)

US9771568B2	Polypeptides having beta-glucosidase activity and polynucleotides encoding same	Polypeptides with BGL activity and polynucleotides encoding enzyme;.nucleic acid constructs, vectors and host cells comprising the polynucleotides as well as methods of producing and using the same.	Novozymes Inc	Liu et al. (2017a)
US9441214B2	Polypeptide having beta-glucosidase activity and uses thereof	Polypeptide comprising the amino acid, or a variant polypeptide or variant polynucleotide thereof; full length coding sequence of the novel gene as well as the amino acid sequence of the full-length functional polypeptide and functional equivalents of the gene or the amino acid sequence. The invention also relates to methods for using the polypeptide in industrial processes.	Versalis SpA	Schoonneveld-Bergmans et al. (2016)
US20220056496A1	Preparation of thermophilic beta-glucosidase and application thereof	Heterologous BGL from the thermophilic fungus *Talaromyces piceae* expressed a recombinant bacteria; the resulting enzyme can produce gentioligosaccharides with a high conversion rate using glucose	Jiangnan University	Wu et al. (2022)
US8143049B2	Modified beta-glucosidases with improved stability	Modified BGL from *Trichoderma reesei* Cel3A with improved stability at low pH, low pH and high aeration, low pH and high agitation, or low pH and elevated temperature; genetic constructs comprising its nucleotide sequences encoding, methods for the production of enzymes from host strains and the use of the enzymes in the hydrolysis of cellulose.	Iogen Energy Corp	Hill et al. (2012)

1.10 Conclusion

BGLs have become one of the most indispensable biocatalysts due to their wide range of industrial applications, and their prominence has become more pronounced with the drive for more sustainable and environmentally friendly fuel alternatives from lignocellulosic biomass. The industrial significance of these enzymes has been established by the quantum of research that has been carried out on the enzyme, some of which were critically reviewed in this research. However, despite the progress made so far, a lot could still be done in the area of catalytic efficiency, end-product tolerance, cost-efficient enzyme production, and effective methods of utilisation. Notable among the future directions for the enzyme include enhanced protein engineering, metabolic engineering, and the use of recent advanced technologies such as Page-assisted continous evolution (PACE), Capsid engineering, CRISPR and base editors which would assist in integrating BGLs into compact metabolic pathways for the bioconversion of biomass into valuable products such as biofuels, as well as various speciality chemicals.

1.11 Research problem, aim and objectives

Enzymes, being the biological catalyst, have become important in various processes in industrial sectors such as food, beverages, textiles, pulp and paper, and bioenergy. β-glucosidase is one of the most important industrial enzymes which are involved in the terminal reactions of the cellulase enzyme system. However, it has been observed that many enzymes currently available for use are only stable and active at a relatively narrow range of reaction conditions. Therefore, the search for a novel enzyme with remarkable characteristics such as higher enzyme activity and stability at extreme temperature and pH conditions has been identified as a worthwhile attempt. In addition to the need for enzymes with improved activity and stability, there is also a high consideration for their safety for human use and their affordability. Hence, this study aimed at investigating a β-glucosidase from *Beauveria bassiana* SAN01, a fungus that has been demonstrated to be safe for human use. Thus, the production of the enzyme was attempted using readily available agricultural residues as the low cost media and the production parameters were optimised via a statistical approach. Furthermore, the purification, biochemical characteristics, and *in silico* analysis of the enzyme was performed to identify and enhance its potential industrial applicability.

1.11.1 Aim

To optimise the production of β-glucosidase from *Beauveria bassiana* SAN01 and evaluate its functional properties, and saccharification potential.

1.11.2 Objectives

- Optimisation of different process parameters for enhanced production of *B. bassiana* SAN01 β-glucosidase using response surface methodology
- Purification of β-glucosidase from *B. bassiana* SAN01 using ammonium sulphate precipitation and chromatographic techniques.
- Biochemical characterisation of *B. bassiana* SAN01 β-glucosidase
- Determination of the saccharification potential of *B. bassiana* SAN01 β-glucosidase
- *In silico* structural characterisation of *B. bassiana* SAN01 β-glucosidase

2. CHAPTER TWO

OPTIMISATION OF THE PRODUCTION OF *BEAUVERIA BASSIANA* SAN01 β-GLUCOSIDASE USING RESPONSE SURFACE METHODOLOGY

Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-024-05866-x

ORIGINAL ARTICLE

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Hyper β-glucosidase producer *Beauveria bassiana* SAN01—optimization of fermentation conditions and evaluation of saccharification potential

Buka Magwaza[1] · Ayodeji Amobonye[1] · Prashant Bhagwat[1] · Santhosh Pillai[1]

Received: 6 March 2024 / Revised: 9 June 2024 / Accepted: 12 June 2024

Abstract

The hyper-production of β-glucosidase by a local strain of *Beauveria bassiana* under submerged conditions is reported in this study. The initial screening of seven agricultural residues showed that the haulm of Bambara—an underutilized African legume—supported the highest β-glucosidase production; hence, statistical optimization of enzyme production was done using this biomass as the sole carbon source. Plackett–Burman design identified the concentrations of Bambara haulm, KCl, and NaCl as well as agitation speed and incubation time as the most significant factors affecting enzyme production. Subsequently, the central composite design predicted the optimal conditions (Bambara 57 g/L, KCl 302 mg/L, NaCl 154 mg/L, agitation speed 150 rpm, and incubation 223 h) for *B. bassiana* β-glucosidase production, which were further validated. The generated quadratic model was deemed significant judging from its *F*-value (201.63), adequate precision ratio (45.74), as well as the R^2 (0.9988), adjusted R^2 (0.9938), and predicted R^2 (0.9195) values. The optimization resulted in a ~5.36-fold increase in enzyme levels from the unoptimized production of ~133 to 711 U/mL. The enzyme was also demonstrated to efficiently hydrolyze cellobiose, converting more than 90% of the substrate to glucose. These results further establish the resourcefulness of the *B. bassiana* strain for the production of β-glucosidase enzyme, having immense potential, especially in the food and energy industries.

Keywords *Beauveria bassiana* · B-glucosidase · Hyper-production · Lignocellulosic biomass · Optimization · Saccharification

This chapter has been published, **Magwaza, B.**, Amobonye, A., Bhagwat, P. and Pillai, S. 2024. Hyper β-glucosidase producer *Beauveria bassiana* SAN01—optimisation of fermentation conditions and evaluation of saccharification potential. *Biomass Conversion* and *Biorefinery*, pp.1-13. https://doi.org/10.1007/s13399-024-05866-x

Abstract

The hyper-production of β-glucosidase by a local strain of *Beauveria bassiana* under submerged conditions is reported in this study. The initial screening of seven agricultural residues showed that the haulm of Bambara- an underutilized African legume- supported the highest β-glucosidase production, hence, statistical optimisation of enzyme production was done using this biomass as the sole carbon source. Plackett Burman design identified the concentrations of Bambara haulm, KCl, and NaCl as well as agitation speed and incubation time as the most significant factors affecting enzyme production. Subsequently, the Central Composite Design predicted the optimal conditions (Bambara 57 g/L, KCl 302 mg/L, NaCl 154 mg/L, agitation speed 150 rpm, and incubation 223 h) for *B. bassiana* β-glucosidase production, which were further validated. The generated quadratic model was deemed significant judging from its F-value (201.63), Adequate Precision ratio (45.74), as well as the R^2 (0.9988), adjusted R^2 (0.9938), and predicted R^2 (0.9195) values. The optimisation resulted in a ~5.36-fold increase in enzyme levels from the unoptimized production of ~133 to 711 U/mL. These results further establish the resourcefulness of the *B. bassiana* strain for the production of β-glucosidase enzyme having immense industrial importance.

2.1 Introduction

Beta-glucosidases (E.C.3.2.1.21) or β-glucosidases or BGLs, are a class of glycosyl hydrolases that catalyse the hydrolysis of β-glycosidic bonds in short-chain oligosaccharides, alkyl- and aryl β-D-glucosides. β-Glucosidases (BGLs) are essential components of the cellulase enzymatic system which also comprises the endoglucanases (E.C.3.2.1.4), and the exoglucanases (E.C.3.2.1.91), which all act in tandem to convert cellulosic material into glucose. However, each enzyme component plays a distinct role in the system; while endoglucanases target the internal areas of amorphous cellulose, exoglucanases act at the reducing and non-reducing ends of the polymer (Bhati et al., 2021). The terminal reaction is catalysed by BGL which hydrolyse the cellobiose (released by the action of endoglucanase and exoglucanases) into glucose monomers (Saroj and Narasimhulu, 2022). Therefore, the efficient hydrolysis of cellulose, especially for the production of biofuels, is facilitated by the enzymatic actions of BGL In addition, the enzymes also possess some side activities that have been explored for other industrial applications (Lv et al., 2022). These activities include their trans-glycosylation potential which serves as the basis of their use in the pharmaceutical industry for oligosaccharide synthesis, hydrolysis of glycosides which is useful in the beverage industries for the improvement of aroma, as well as the production of alkyl glucosides that are useful in cosmetics and detergents production (Kannan et al., 2023).

With the global shift towards cleaner energy production as highlighted in the UN Sustainable Developmental Goal 7, BGL have become prominent among the most sought-after enzymes. Consequently, BGL were estimated to account for one-quarter of the global agricultural enzymes market, valued at ~USD 380 million in 2021 and projected to reach ~USD 760.23 million by 2027 (360iResearch, 2021). BGL from several fungal genera such as *Aspergillus* (El-Ghonemy, 2021), *Fusarium* (Bertonha et al., 2018), and *Trichoderma* (Sun et al., 2022) have been demonstrated for their significant functional properties and their suitability for industrial usage. However, one of the major bottlenecks for the utilization of BGLs in the industry is their high production cost, resulting into high overhead costs. Thus, the possible approaches to address this constraint include finding novel isolates capable of utilizing low-cost media for enzyme production, optimizing fermentation conditions for maximal enzyme release as well as genetic engineering of the strain for heightened BGL productivity and improved functionality.

In the last five decades, various scientific attempts – including the recent ones- have been made at addressing the challenges mentioned above (MuleSawant and Odaneth, 2024, Senba et al.,

2023, SunWang and Hao, 2024). For instance, various novel microbial strains have been demonstrated to utilize different readily available growth substrates for BGL production, such as the fungi, *Aspergillus tubingensis* and *Trichoderma reesei* which utilized maize bran (MuleSawant and Odaneth, 2024), wheat bran (Frassatto et al., 2021), respectively as well as the actinomycetes *Jiangella alba* which utilized wheat straw for the same purpose(Aytaş et al., 2023). Similarly, BGL genes from numerous microbes have been successfully cloned and expressed in efficient host systems for increased enzyme yields and ease of purification. In the study by Senba et al., (2023), BGL gene from *Aspergillus chevalieri* MK86 was expressed in *Aspergillus oryzae* to achieve a 100-fold increase in production relative to the native organism. Similarly, a recombinant BGL with a 9-fold increased production level was obtained when the gene from *Devosia* sp. Arc12 was expressed in *Escherichia* coli BL21 (DE3) (SunWang and Hao, 2024). However, like for all other industrially important enzymes, the search for the optimum microbial source that would overcome one or more of the current limitations hindering the significant utilization of BGL in the industry continues (Golgeri M et al., 2024).

Recently, a strain of the entomopathogenic fungal endophyte, *Beauveria bassiana* was discovered as a novel source of industrially important enzymes. Typically *B. bassiana* is used in the formulation of biopesticides; it was also highlighted for its ability to produce various bioactive compounds such as beauvericin, bassianin, beauveriolides, tennelin and oosporein (Amobonye et al., 2020). The fungus is non-pathogenic to humans, animals, and plants (Zimmermann, 2007); hence, it is considered safe for human use as well as for the environment. *B. bassiana* SAN01 was observed to proliferate rapidly on various lignocellulosic biomass while simultaneously secreting significant quantities of lignocellulosic degrading enzymes, making it a potential mining site for industrial carbohydrases (Amobonye et al., 2023). It was further observed during preliminary screening that the fungus' BGL production level under unoptimised conditions was higher than the previously reported for any *Beauveria* strain or other entomopathogenic fungal endophyte.

Process parameter optimisation has been considered critical in the quest for cost-effective and enhanced enzyme production as parameters such as temperature, pH, fermentation time, and media components profoundly affect enzyme production (Dhaver et al., 2022). Historically, the optimisation of fermentation processes entails the systematic alteration of an individual independent variable or factor, while maintaining the other variables at constant values; a process that is quite slow and labour-intensive, especially when dealing with a substantial

number of independent variables (Nisar et al., 2020). However, the conventional optimisation approach fails to account for the interplay between the variables utilized and their overall impact on the required output, hence, there is a growing preference for statistical techniques such as Response Surface Methodology (RSM) in the optimisation of fermentation processes (Breig and Luti, 2021). This statistical approach decreases the overall number of required experiments and better elucidates the relationships between various elements and the fermentation output (Singh et al., 2023b). RSM includes Plackett-Burman design (PBD) which efficiently identifies the most influential fermentation components out of many and the Central Composite design (CCD) which ascertains the ideal concentration of individual components, evaluates their interaction, and also assesses their impact on the response (Baskaran and Krishnan, 2020).

Thus, *B. bassiana* SAN01, a lignocellulosic-degrading strain of *B. bassiana,* was investigated for its potential to serve as an industrial source of BGL based on the safety profile of the entomopathogenic fungal endophyte, as well as its notable industrial importance. In this regard, the study was aimed at maximizing the production of BGL by *B. bassiana* SAN01 using readily available agricultural residues under submerged fermentation. To achieve this, RSM at two levels was employed to optimize the fermentation parameter involved in *B. bassiana* BGL production, followed by the validation of the derived model. In addition, the efficiency of the BGL in cellobiose hydrolysis was also assessed in this study. To the best of our knowledge, this is the first attempt at the production of BGL by any *Beauveria* strain using lignocellulosic biomass as the substrate and optimizing the process via a statistical approach.

2.2 Methodology

2.2.1. Materials

The lignocellulosic biomass used in this study was obtained locally in Durban, South Africa. Chemicals including 4-nitrophenyl β-D-glucopyranoside (pNPG), were purchased from Sigma-Aldrich (South Africa). All other reagents used in this study were of HPLC or analytical grade. The Design Expert 11.0 (Stat-Ease, Minneapolis, USA) software was used for both the first (Plackett- Burman design) and second (Central Composite design) level optimisation as well as for the validation of the generated model.

2.2.3 *B. bassiana* SAN01 culture conditions

The endophytic fungus *Beauveria bassiana* (Gene Accession Number: MN544934) selected for the production of β-glucosidase was obtained from the culture collection of the Department

of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa. The fungus was grown on potato dextrose agar (PDA) at 30°C for five days. Afterwards, *B. bassiana* spore suspension (1 x 10^6 spores/mL) was prepared following the protocol of Amobonye et al. (2021).

2.2.4. Screening of lignocellulosic biomass as the main carbon source

B. bassiana SAN01 BGL production was evaluated using seven different biomass samples (aspen wood, Bambara haulm, corn cob, molasses bran, sugarcane bagasse, wheat bran, and wheat straw). Fermentation was carried out in 250 mL Erlenmeyer flasks with 100 mL mineral salt solution containing (g/L) $(NH_4)_2SO_4$ (4.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.5), KCl (0.5), NaCl (0.25), $CaCl_2$ (0.2), $FeSO_4$ (0.1) and 40 g/L of the respective biomass at pH 6.0. The flask was inoculated with 1 mL of spore suspension (1 x 10^6 spores/mL) and incubated for 9 days at 30°C and 150 rpm. Afterwards, the culture broth was filtered using a sterile muslin cloth and centrifuged for 15 min at 4°C and 10,000 x g. The supernatant retrieved after centrifugation was used as the crude enzyme for subsequent experiments.

2.2.5 Enzyme assay

β-D-glucopyranoside (pNPG) was used as the chromogenic substrate for the quantification of *B. bassiana* SAN01 β-glucosidase activity. The reaction mixture comprised of 0.9 mL of pNPG (5 mM) and 0.1 mL of the appropriately diluted enzyme, incubated at 60°C for 10 min. The reaction was terminated by the addition of 1 mL of Na_2CO_3 (0.5 M), and absorbance was read at 410 nm using a UV-Vis spectrophotometer (Shimadzu UV- 1900i). One unit of β-glucosidase activity was defined as the amount of enzyme that generated 1 µmol of p-nitrophenol in 1 min at 60°C (da Costa et al., 2018). The protein content of the samples was also determined by a modified Lowry method using bovine serum albumin as the standard (Hartree, 1972).

2.2.6 Statistical optimisation of the fermentation conditions

2.2.6.1 Plackett- Burman design (PBD)

The screening of the fermentation media components to identify the ones with significant effects on the production level of *B. bassiana* SAN01 BGL was conducted using PBD (Sorour et al., 2023). This approach employs a linear methodology for identifying the important components while disregarding any potential interactions among the components following the first-order polynomial as represented in Eqn. (1).

$$Y = \beta 0 + \Sigma \beta i Xi \quad (1)$$

Y = mean of BGL activity; β0 = intercept of the model; βi = linear coefficient; Xi = the value of an independent component.

Optimisation at this level involved a total of 11 components, consisted of nine production media components and two physical components, which were selected from preliminary studies and previous literature (Sun et al., 2021, Bhaturiwala et al., 2022). The experimental design included 13 runs, with 1 centre point. The analysis of each component within the medium was evaluated at high (+1) and low (-1) levels; the real and coded range of the investigated factors are summarised in Table 2.1. The experiments were conducted in triplicate, and the mean value of BGL activity (U/mL) served as the measured response. The components in the production medium that exhibited confidence levels over 95% were deemed significant to BGL production and were subsequently optimised in CCD.

Table 2.1: The experimental variables and actual levels used in Plackett-Burman design

Variable	Unit	Symbol	Coded value		
			-1	0	+1
Bambara	(g/L)	A	20	40	60
$(NH_4)_2SO_4$	(g/L)	B	2	4	6
KH_2PO_4	(mg/L)	C	800	1000	1200
KCl	(mg/L)	D	400	500	600
$MgSO_4$	(mg/L)	E	400	500	600
NaCl	(mg/L)	F	200	250	300
$CaCl_2$	(mg/L)	G	150	200	250
$FeSO_4$	(mg/L)	H	80	100	120
Agitation speed	rpm	I	140	150	160
pH	-	J	5.5	6	6.5
Incubation time	h	K	168	192	216

2.2.6.2 Central composite design (CCD)

CCD was utilized to determine the optimal values of the selected factors obtained from PBD analysis *viz*., concentrations of the carbon source (Bambara haulm), KCl, and $CaCl_2$ as well as the incubation time and agitation speed. The five selected variables were investigated at five coded levels (-β, -1, 0, 1, β) with Table 2.2. Summarising their actual and coded range values. The remaining eight components were kept at values equivalent to the central point values employed in the PBD. All runs were conducted in triplicates and the response was the calculated average BGL activity. The relationships among the variables were established via a second-order polynomial equation as represented in Eq. (2).

$$Y = \beta 0 + \Sigma \beta i Xi + \Sigma \beta ii Xi^2 + \Sigma\, i \Sigma \beta ij Xi\, Xj \quad (2)$$

Y = mean of BGL activity; Xi and Xj = independent components; β0 = intercept constant, βi = linear coefficients; βii = quadratic coefficient; βij = coefficient of two-component interaction. Each variable in the equation was fitted to determine its significance and an ANOVA (analysis of variance) test was done to observe the goodness of fit. The interactions among the evaluated components were represented and visualized in contour plots (Boro and Verma, 2023a).

Table 2.1: The range of significant components used in CCD

Variables	Symbol	-β	-1	0	+1	+β
Bambara (g/L)	A	20	31	45	58	70
KCl (mg/L)	B	200	290	400	509	600
NaCl (mg/L)	C	100	145	200	254	300
Agitation speed (rpm)	D	140	150	160	170	180
Incubation time (h)	E	168	184	204	223	240

Furthermore, to validate the model and the optimal concentration of the variables (A, B, C, D, and E) required for maximal *B. bassiana* BGL production, the experiments were performed with the values predicted by the model; finally, correlation analysis was performed on the predicted and obtained response values for each solution (Boro and Verma, 2023b).

2.2.7 Statistical analyses

Statistical variance (ANOVA) was calculated with GraphPad Prism software (version 10) and data were all presented as the mean ± standard deviation of triplicate values. Differences between samples were undertaken to be statistically significant if $p < 0.05$.

2.3 Results and discussion

2.3.1 Valorisation of lignocellulosic biomass for β-glucosidase production

In this study, *B. bassiana* SAN01 was observed to proliferate remarkably on different lignocellulosic biomass and produce BGL in high quantities. Amongst the biomass tested, Bambara haulm and wheat bran were observed to be the most promising, supporting higher β-glucosidase yields of 132.71 U/mL and 87.80 U/mL, respectively (Fig 2.1). The significant production level of BGL recorded with Bambara haulm in this study is the first report utilizing the agricultural residue for BGL production. Bambara is one of the underutilized pulse crops in Africa, however, the residue after harvest (the haulm) is usually burnt on the farm or used as fodder for livestock animals (Majola et al., 2021).However, recent studies have shown that it contains a significant quantity of cellulose (37%) and hemicellulose (15%) (Okuofu et al., 2022), hence it possesses the potential for valorisation, which was successfully explored in this study for *B. bassiana* BGL production. Although there is currently no information detailing the underlying factors behind the efficient utilization of Bambara haulm as a fermentation substrate, we hypothesize that being a leguminous plant, it contains an adequate amount of required nutrients to support robust fungal metabolism and enzyme production. Furthermore,

as observed in wheat bran and other lignocellulosic biomass, its constituent biopolymers such as cellulose and hemicellulose, can upregulate the expression of relevant genes, leading to significant enzyme production. Wheat bran has been the ideal carbon source for industrial biocatalysts such as microbial amylases, cellulases, and xylanases (Abdeshahian et al., 2020, Kumar et al., 2018). Various studies have described the efficient utilization of wheat bran by fungi for BGL production including the recent 812 U/mL attained by *Aspergillus versicolor* (Huang et al., 2021).

However, in this study, it stood as the second most important biomass for the BGL production after Bambara. On the other hand, aspen wood shaving and sugarcane bagasse could not support *B. bassiana* SAN01 BGL production despite their high carbon content. For example, sugarcane bagasse comprises 40-50% cellulose, 25-35% hemicellulose, and 5-15% lignin (Infanzón-Rodríguez et al., 2023), however, its low nitrogen content might be responsible for its inability to promote enzyme production in this study; as well as aspen wood shavings. Interestingly, it was remarkable to observe that the unoptimised BGL production level obtained from *B. bassiana* SAN01 in this study is 25.38-folds higher than the 5.20 U/mL from another strain of *Beauveria* (Borgi et al., 2016). Bambara haulm being the best biomass for BGL production by *B. bassiana* SAN01 was further used for optimisation using RSM.

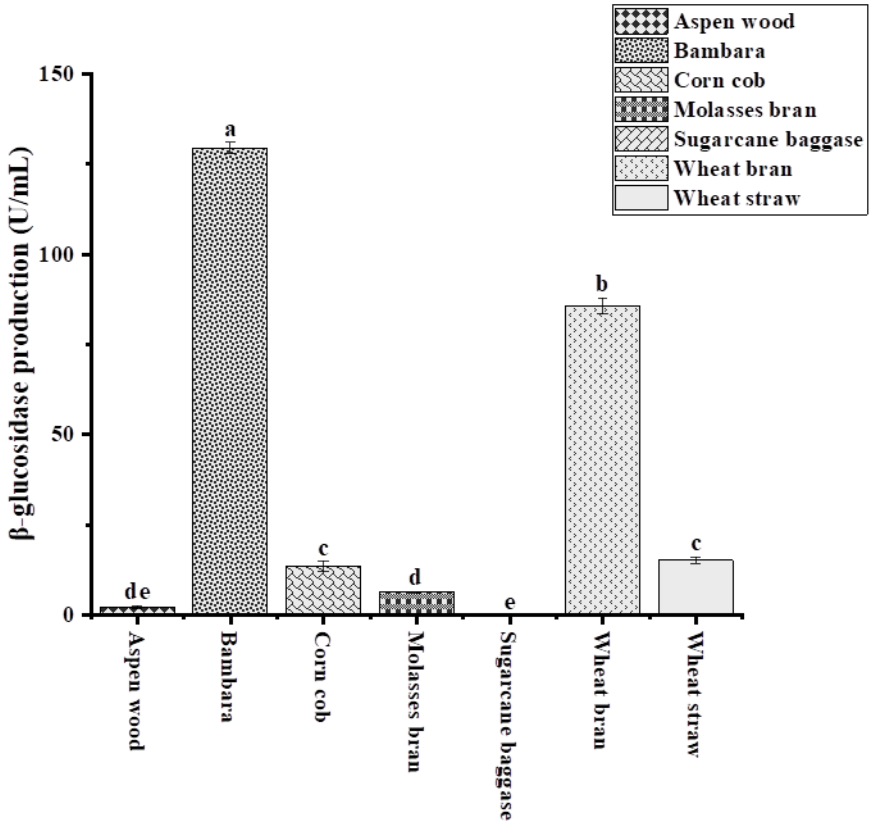

Figure 2.1: *B. bassiana* SAN01 β-glucosidase production levels using different lignocellulosic biomass (Bars with the same letter superscripts are not significantly different at $p< 0.05$)

2.3.2 Screening of significant variables by Plackett- Burman design

In this study, a 13-PBD run was utilized to screen the fermentation factors with significant effects on *B. bassiana* SAN01 BGL production. The outcomes of the first level optimisation are summarised in Table 2.3; the experiments showed that five factors possessed significant effects on enzyme production. In this regard, the statistical significance of these effects is illustrated on the Pareto chart (Fig. 2.2); which highlights the concentration of Bambara, KCl, and NaCl as well as the incubation time and agitation speed as the critically important parameters influencing the production of the BGL. Similarly, coefficient estimates also demonstrate the correlation and weight of each highlighted factor to BGL production (appendix 1). Furthermore, production levels of between 157.14 and 527.86 U/mL were observed in real-time; while the highest observed and predicted production levels were recorded to be 527.86 and 511.57 U/mL, respectively. The p-value < 0.05 indicates the significance of the corresponding factors, with the incubation time, KCl, NaCl, Bambara concentration, and agitation speed with p-values ranging from < 0.0001 to 0.0062 established as the most significant factors (Table 2.4). These results are in agreement with the previous studies that

identified incubation time (Singh et al., 2023c, Nisar et al., 2020), and agitation (Singh et al., 2023c, Huang et al., 2021) as significant factors in BGL production by different microorganisms. However, a negative correlation between KCl concentration and enzyme production was observed in this study; this is in contrast to a study on *Aspergillus versicolor,* where the same salt displayed a positive relationship with BGL production (Huang et al., 2021). Furthermore, the *F*-value of 36.86 recorded in this study implies the model was significant, and there is only a 0.01% chance that the *F*-value could result from noise. The predicted R^2 of this model was recorded to be 0.8792, which was in reasonable agreement with the adjusted R^2 of 0.9442 (Table 2.4). Thus, the first-order regression equation showed BGL activity as a linear function of the concentrations of Bambara (A), KCl (D), and NaCl (F) as well as agitation (J) and incubation time (L).

BGL production level = 292.19 + 101.72A -46.57D - 41.29F + 39.92J + 64.95L

Hence, the ANOVA analysis and regression coefficients all established that the predicted model for *B. bassiana* SAN01 BGL production was sufficient to annotate the significance of the five chosen factors, thus, these factors were utilized subsequently in CCD for the optimisation of the response and the curvature of *B. bassiana* SAN01 BGL yield.

Table 2.3: Plackett-Burman design and the mean responses for β-glucosidase production

Run	A	B	C	D	E	F	G	H	I	J	K	Production level (U/mL)	
												Observed	Predicted
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	520.6	508.9
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	413.6	463.6
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	157.1	178.2
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	527.9	511.6
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	169.1	210.2
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	176.2	204.9
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	210.1	223.5
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	203.3	175.5
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	341.4	296.5
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	216.1	212.9
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	187.1	164.1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	226.2	204.9
13	0	0	0	0	0	0	0	0	0	0	0	499.1	498.3

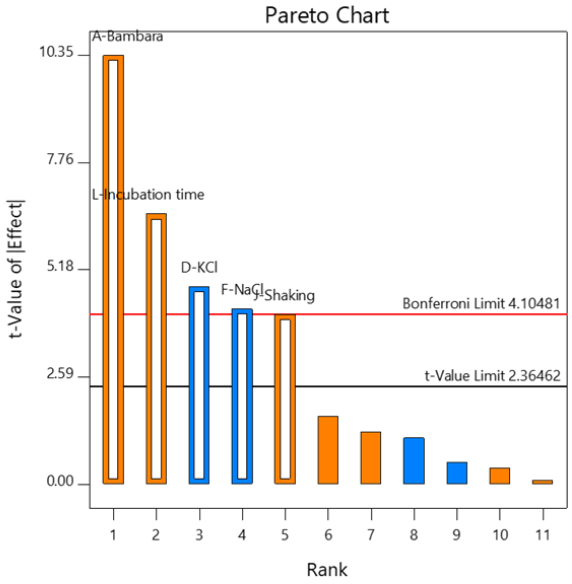

Figure 2.2: The Pareto chart of standardised effects for the production of *Beauveria bassiana* SAN01 β-glucosidase (the orange and blue colours represent positive and negative effects respectively)

Table 2.4: ANOVA of Plackett-Burman design for *Beauveria bassiana* SAN01 β-glucosidase activity

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	2.404E+05	5	48077.00	36.24	< 0.0002	significant
A-Bambara	1.242E+05	1	1.242E+05	98.75	< 0.0001	
D-KCl	26019.86	1	26019.86	20.69	0.0039	
F-NaCl	20453.61	1	20453.61	16.27	0.0069	
I-Agitation	19128.06	1	19128.06	15.21	0.0080	
K-Incubation time	50616.59	1	50616.59	37.86	0.000	
Residual	7544.36	6	2232.30	40.26		
Cor Total	2.502E+05	12	1257.39			

C.V% = 11.98, R^2 = 0.9696, Adjusted R^2 = 0.9442, Predicted R^2 = 0.8792 and Adequate precision- 13.3210

2.3.3 Optimisation by central composite design and model validation

The optimal concentration of the significant factors obtained from PBD was determined using CCD for maximum BGL production, the 2^3 full factorial design was also utilized to study the interactions between the selected factors. The 26-run experiments and the observed output are depicted in Table 2.5. In addition to the data presented in the table, the actual vs predicted plots also elucidate the close agreement between the experimental and predicted output, further highlighting the significance of RSM in the optimisation of fermentation conditions for enhanced *B. bassiana* SAN01 BGL production (appendix 4). The analysis of the coefficient estimates of the CCD revealed the positive linear effects of incubation time, Bambara concentration, and agitation and the negative effects of KCl and NaCl concentrations on BGL production (appendix 6). The observed experimental values vary from 258.7 to 711 U/mL (Table 2.5). ANOVA results suggested the adequacy of the second-order response, in this regard, the model *F*-value of 201.63 indicates the model's significance. The goodness of fit of the model was illustrated by the reasonable agreement between the Predicted R^2 (0.9195) and the Adjusted R^2 (0.9938) (Table 2.6). Adeq Precision is a measure of the signal-to-noise ratio, with a value greater than 4 being desirable (Amin et al., 2021, Baskaran and Krishnan, 2020). Hence, the obtained Adeq Precision ratio of 45.74 in this study indicates an adequate signal; consequently, the model can be used to navigate the design space (Baskaran and Krishnan, 2020). Furthermore, the factors both in their individual and interactive capacities- A, B, C, D,

E, AB, BC, BE, CD, CE, DE, A^2, D^2, and E^2 were recorded with p-values < 0.05, thus they were all deemed to be significant and reliable.

The regression equation derived from the ANOVA demonstrated that BGL production is a function of A concentration of Bambara, B - KCl concentration, C - NaCl concentration, D - agitation, and E - incubation time. Thus, the model quadratic regression is given as:

BGL activity = 515.41 + 05.15A -28.33 B - 32.41C - 55.82D + 20.53E +21.97AB + 6.94AC +15.06 AD + 7.63AE - 17.80BC -9.61 BD - 39.96BE - 25.03CD - 54.56CE -37.10DE -19.12 A^2 - 4.11B^2 -2.35 C^2 +11.03 D^2 -38.12 E^2

Table 2.5: Experimental design in terms of actual variables and response of the Central composite design (CCD)

Run	Bambara (A)	Incubation time (E)	Agitation speed (D)	KCl (B)	NaCl (C)	BGL Production level (U/mL)	
						Observed	Predicted
1	+1	-1	+1	+1	-1	574.8	573.8
2	+1	-1	+1	-1	+1	534.9	533.9
3	-1	+1	-1	+1	+1	312.2	311.2
4	+1	-1	-1	+1	+1	623.9	622.9
5	+1	+1	-1	+1	-1	711.0	710.0
6	+1	+1	+1	-1	-1	662.4	661.4
7	-1	+1	+1	-1	+1	262.2	261.2
8	-1	+1	+1	+1	-1	300.4	299.4
9	+1	+1	-1	-1	+1	674.4	673.4
10	-1	-1	+1	0	+1	264.3	263.4
11	-1	-1	-1	0	-1	323.1	321.2
12	-β	0	0	-β	0	258.7	260.5
13	+β	0	0	+β	0	641.7	643.5
14	0	0	0	0	0	551.6	553.4
15	0	0	0	0	0	448.4	450.2
16	0	0	0	0	-β	564.9	566.7
17	0	0	0	0	+β	446.8	448.6
18	0	0	-β	0	0	651.9	653.7
19	0	0	+β	0	0	448.5	450.3
20	0	-β	0	0	0	349.8	351.6
21	0	+β	0	0	0	424.6	426.4
22	0	0	0	0	0	523.2	515.4
23	0	0	0	0	0	527.2	515.4
24	0	0	0	0	0	508.4	515.4
25	0	0	0	0	0	524.1	515.4
26	0	0	0	0	0	500.3	515.4

The Box-Cox plot shows the optimal transformation to normalize a set of data that is not normally distributed by specifying an appropriate Lambda (λ) for the model; hence it was evaluated to determine if any transformation was required to improve the normality of residuals. In this study, the Box-Cox graph for power transforms presented a Lambda value of 1 for the response variable, hence, demonstrating that the response does not require any transformation for *B. bassiana* BGL production (appendix 5). Thus, three-dimensional response surface plots were generated based on the CCD quadratic regression to demonstrate the pair-wise interactive effects of two variables and their effect on *B. bassiana* SAN01 BGL production. The z-axis in the 3D response surface plots represents the activity of BGL as it relates to two specific variables while holding all other variables constant (Fig. 2.3). Interactions between AB, BE, CD, CE, and DE were observed to be significant with p-value < 0.05; while the interactions between AC, AD, AE, BC, and BD were deemed to be insignificant due to p-value > 0.05 Table 2.6. *B. bassiana* SAN01 BGL production was observed to increase with increasing concentration of Bambara and decreasing concentration of KCl (Fig. 2.3.). The interaction between NaCl and KCl as illustrated in Fig. 3b showed that BGL production was favoured at lower concentrations of both NaCl and KCl; while BGL production decreased as both the concentrations were steadily increasing corroborating their negative effects on the BGL production. Interaction between incubation time and all other tested variables indicated that as incubation time increased, BGL production also increased, reaching a maximum level at 223 h, however, a gradual decline in enzyme production levels was observed beyond the optimum incubation time (Fig. 2.3. c, e and f). The decline in BGL production after 223 h could be attributed to nutrient depletion which affected *B. bassiana* SAN01 metabolism or the accumulation of proteases and other byproducts resulting in BGL degradation as well as cell autolysis (Akula and Golla, 2020). These results are corroborated by previous studies where enzyme production was also noted to have steeply declined beyond the optimum incubation time (Nisar et al., 2020, Singh et al., 2023b). Interactions between incubation time and NaCl (CE) was more statistically significant amongst all other tested interactions in this study with the observed p-value of 0.0003 (<0.05) (Fig. 2.3. e). In addition, from the contour plots graphs, agitation between 140-150 rpm and NaCl concentration between 150-200 (mg/L) resulted in higher BGL production (Fig. 2.3. d). According to literature and the results obtained in this study, it can be inferred that further increase in BGL production in different microorganisms is possible by optimisation of medium components and physical components via statistical methods, respectively (Dhaver et al., 2022, Huang et al., 2021, Singh et al., 2023c).

Table 2.6: Analysis of variance (ANOVA) for CCD experimental results

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4.822E+05	20	24108.31	201.63	< 0.0001	significant
A-Bambara	73340.67	1	73340.67	613.38	< 0.0001	
B-KCl	5322.67	1	5322.67	44.52	0.0011	
C-NaCl	6968.64	1	6968.64	58.28	0.0006	
D-Shaking	20671.54	1	20671.54	172.89	< 0.0001	
E-Incubation time	2796.12	1	2796.12	23.39	0.0047	
AB	1474.65	1	1474.65	12.33	0.0171	
AC	147.01	1	147.01	1.23	0.3180	
AD	693.58	1	693.58	5.80	0.0610	
AE	177.72	1	177.72	1.49	0.2772	
BC	967.84	1	967.84	8.09	0.0360	
BD	282.22	1	282.22	2.36	0.1851	
BE	4879.76	1	4879.76	40.81	0.0014	
CD	1915.16	1	1915.16	16.02	0.0103	
CE	9099.22	1	9099.22	76.10	0.0003	
DE	4207.49	1	4207.49	35.19	0.0019	
A^2	7574.12	1	7574.12	63.35	0.0005	
B^2	349.53	1	349.53	2.92	0.1480	
C^2	114.38	1	114.38	0.9567	0.3730	
D^2	2521.31	1	2521.31	21.09	0.0059	
E^2	30109.61	1	30109.61	251.82	< 0.0001	
Residual	597.84	5	119.57			
Lack of Fit	52.80	1	52.80	0.3875	0.5673	not significant
Pure Error	545.04	4	136.26			
Cor Total	4.828E+05	25				

C.V% = 2.25, R^2= 0.9988, Adjusted R^2= 0.9938 , Predicted R^2= 0.9195 and Adequate precision- 45.74

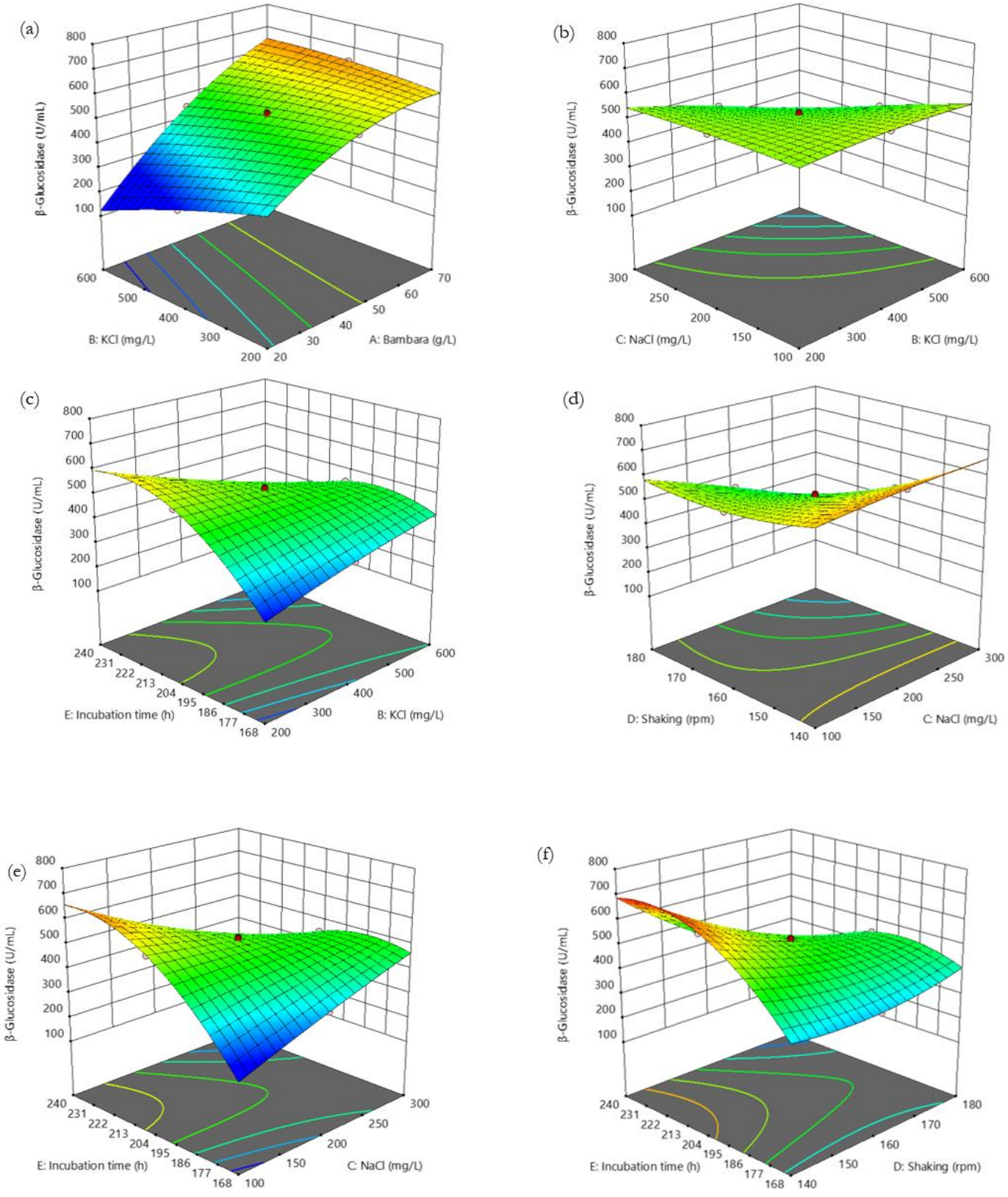

Figure 2.3:3D-response surface plots for *B. bassiana* SAN01 β-glucosidase activity (a) effect of KCl versus Bambara concentration,(b) effect of KCl versus the NaCl concentration (c) effect

of incubation time versus KCl concentration (d) effect of agitation speed versus NaCl concentration

To validate the derived factorial model, the optimal values of the production factors predicted by CCD were concentrations of Bambara (57.03 g/L), KCl (302.81 mg/L), and NaCl (154.79 mg/L), as well as agitation speed of 150 rpm, and incubation time of 223 h. The BGL activity obtained with this predicted conditions was 711.0 U/mL which was in close agreement with predicted values of 710.4 U/mL. This significant correlation between the predicted and obtained values demonstrates the adequate accuracy of RSM in the optimisation of the fermentation parameters during *B. bassiana* SAN01 BGL production as well as validates the existence of optimum points. Through this statistical optimisation approach, a 5.39-fold increase was recorded in BGL production relative to the unoptimised level of 132.71 U/mL. These results are remarkable as the optimised BGL yield recorded in this study is the highest level ever recorded from any entomopathogenic or endophytic fungus, thus, unlocking the potential of *B. bassiana* SAN01 as a viable source of BGL.

2.4 Conclusion

This study successfully optimised the production of BGL by the novel endophytic fungus *B. bassiana* SAN01 via statistical modelling using PDB and CCD in submerged fermentation. An acceptable level of agreement was found between the experimental results and the model. BGL production improved by 5.39-fold compared to unoptimised level and according to our knowledge, the BGL activity obtained in this study is the highest level ever recorded from any entomopathogenic or endophytic microbe. Thus, these results demonstrate the potential of statistical methods in optimizing the production medium for BGL production by *B. bassiana* SAN01 while utilizing lignocellulosic biomass. However, it is considered important that further studies are required to elucidate the mechanism behind the significant production of BGL with Bambara haulm as the carbon source, both at the micro level and molecular level. Furthermore, it is imperative to upscale the production of *B. bassiana* SAN01 BGL to the bioreactor scale, all with the aim of enhancing its real-time industrial applications.

3 CHAPTER THREE

PURIFICATION, BIOCHEMICAL AND *IN SILICO* STRUCTURAL PROPERTIES OF A THERMO-ACID STABLE β-GLUCOSIDASE FROM *BEAUVERIA BASSIANA* SAN01

Heliyon 10 (2024) e28667

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Biochemical and *in silico* structural properties of a thermo-acid stable β-glucosidase from *Beauveria bassiana*

Buka Magwaza, Ayodeji Amobonye, Prashant Bhagwat, Santhosh Pillai *

Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, P. O. Box 1334, Durban, 4000, South Africa

ARTICLE INFO

Keywords:
β-glucosidase
Beauveria bassiana
Enzyme purification
In silico
Molecular docking
Structural elucidation

ABSTRACT

β-glucosidase hydrolyses the glycosidic bonds in cellobiose and cello-oligosaccharides, a critical step in the saccharification for biofuel production. Hence, the aim of this study was to gain insights into the biochemical and structural properties of a β-glucosidase from *Beauveria bassiana*, an entomopathogenic fungus. The β-glucosidase was purified to homogeneity using salt precipitation, ultrafiltration, and chromatographic techniques, attaining a specific activity of 496 U/mg. The molecular mass of the enzyme was then estimated via SDS-PAGE to be 116 kDa, while its activity pattern was confirmed by zymography using 4-methylumbelliferyl-β-D-glucopyranoside. Furthermore, the pH optima and temperature of the enzyme were found to be pH 5.0 and 60 °C respectively; its activity was significantly enhanced by Mg^{2+} and Na^{+} and was found to be relatively moderate in the presence of ethanol and dichloromethane. Molecular docking of the modelled *B. bassiana* β-glucosidase structure with the substrates, *viz.*, 4-nitrophenyl β-D-glucopyranoside and cellobiose, revealed the binding affinity energies of −7.2 and −6.2 (kcal mol^{-1}), respectively. Furthermore, the computational study predicted Lys-657, Asp-658, and Arg-1000 as the core amino acid residues in the catalytic site of the enzyme. This is the first investigation into a purified β-glucosidase from *B. bassiana*, providing valuable insights into the functional properties of carbohydrases from entomopathogenic fungal endophytes.

This chapter has been published: **Magwaza, B**., Amobonye, A., Bhagwat, P. and Pillai, S. 2024. Biochemical and *in silico* structural properties of a thermo-acid stable β-glucosidase from *Beauveria bassiana*. Heliyon: 10 (7), e28667. https://doi.org/10.1016/j.heliyon.2024.e28667

ABSTRACT

β-glucosidase hydrolyses the glycosidic bonds in cellobiose and cello-oligosaccharides, a critical step in the saccharification for biofuel production. Hence, the aim of this study was to gain insights into the biochemical and structural properties of a β-glucosidase from *Beauveria bassiana* SAN01, an entomopathogenic fungus. The β-glucosidase was purified to homogeneity using salt precipitation, ultrafiltration, and chromatographic techniques, attaining a specific activity of 496 U/mg. The molecular mass of the enzyme was then estimated via SDS-PAGE to be 116 kDa, while its activity pattern was confirmed by zymography using 4-methylumbelliferyl-β-D-glucopyranoside. Furthermore, the pH optima and temperature of the enzyme were found to be pH 5.0 and 60°C respectively. The suitability of the *B. bassiana* SAN01 β-glucosidase in the industry was also evaluated with the enzyme efficiently hydrolysing cellobiose into glucose, converting more than 90% of the substrate to glucose. Furthermore, molecular docking of the modelled *B. bassiana* SAN01 β-glucosidase structure with the substrates, *viz*., 4-nitrophenyl β-D-glucopyranoside and cellobiose, revealed the binding affinity energies of -7.2 and -6.2 (kcal mol^{-1}), respectively. Moreover, the computational study predicted Lys-657, Asp-658, and Arg-1000 as the core amino acid residues in the catalytic site of the enzyme. This is the first study that purified β-glucosidase from *B. bassiana* SAN01, to homogeneity thus, providing valuable insights into the functional properties of carbohydrases from entomopathogenic fungal endophytes.

3.1 Introduction

β-glucosidase (BGL) [E.C.3.2.1.21] is a vital component of the cellulase enzyme system that hydrolyses glycosidic bonds in cellobiose and cello-oligosaccharides into the monomeric unit, glucose. The enzyme is widely distributed across nature, serving various biological functions in animals, bacteria, fungi, and plants. In bacteria and fungi, BGL catalyses the degradation of polysaccharides for energy and plant cell wall metabolism while also playing important roles in their pathogenic and symbiotic associations (Singh et al., 2016). In plants, however, BGL is vital in cellular growth, signalling, and metabolism; intermediate lignification and phytohormone activation; as well as defence against pathogens and other microbial interactions (Godse et al., 2021). Thus, BGLs act on a wide variety of substrates, hence, they are classified based on their substrate specificity into aryl BGLs, cellobiosases, and wide-range BGLs (Liew et al., 2018b). BGLs are noted to be indispensable in various industries, especially in the energy industry during cellulosic biofuel production, as well as in the food industry during winemaking and biosynthesis of flavonoid aglycones from glycosides (Nair et al., 2013).

With the global shift towards cleaner energy production, as highlighted in the United Nations Sustainable Developmental Goal 7, BGLs have become one of the most sought-after enzymes as they catalyse the rate-limiting step of cellulose hydrolysis (Godse et al., 2021). It has been established that the efficient saccharification of cellulose is unachievable without the action of BGLs (which also removes the feedback inhibition of cellobiose), in synergy with endoglucanase and exoglucanases (Singh et al., 2016). In this regard, there is a continuous search for BGLs with more robust properties that would enhance their applicability in the relevant bioprocesses. These desired properties include but are not limited to thermostability, pH stability, and stability in the presence of various chemical additives and solvent systems. To this end, BGLs from different microbial species, especially fungi, such as *Aspergillus* species (Auta et al., 2016), *Penicillium* species (da Costa *et al*., 2018), *Fusarium* species (Bertonha et al., 2018), have been evaluated for their potentials in different industries. However, for an efficient enzymatic system, there has to be a balance between the safety of the source-organism, the functional characteristics of the synthesized enzymes as well as the cost of enzyme production.

Recent findings have shown that a strain of *Beauveria bassiana*, an entomopathogenic fungal endophyte, can secrete several cellulose/hemicellulose degrading enzymes including BGL, in high quantities while utilising different agricultural waste biomass as its growth substrate (Amobonye et al., 2021). In addition, *B. bassiana*, has also been shown to be non-pathogenic

to humans, as well as to plants and animals (Keswani et al., 2013). In this study, an extracellular BGL produced by *B. bassiana* SAN01 under submerged fermentation conditions was purified to homogeneity via precipitation, ultrafiltration, and chromatography and subjected to biochemical characterisation. Furthermore, due to the paucity of data on the structure of glycosyl hydrolases from *B. bassiana, in silico* approach was employed in this study to gain some valuable insights into the enzyme structure. Computational structural analyses have been established to be inexpensive, offering insightful information about the interactions, structure, and function of proteins as well as the various biological processes in which they are involved (Amobonye et al ., 2022).In this regard, the primary, secondary, and tertiary structures of BGL were studied to predict the possible functions associated with the enzyme active site, which may also serve as the basis for future work such as genetic manipulation, enzyme crystallography, and protein engineering. According to available literature, this is the first study aimed at identifying the functional properties of a homogenously purified β-glucosidase from an entomopathogenic fungal endophyte for future industrial applications.

3.2 Methodology

3.2.1 Materials

All the chemicals and reagents used in this study were of analytical grade and procured from Sigma Aldrich, USA. The culture media were purchased from Thermo Fisher Scientific Inc., USA while the lignocellulosic substrate, Bambara haulm, was sourced locally from Durban, South Africa

3.2.2 *B. bassiana* SAN01 culture conditions

Beauveria bassiana SAN01 (Gene Accession Number: MN544934), obtained from the culture collection of the Department of Biotechnology and Food Science, Durban University of Technology was grown on potato dextrose agar (PDA) at 30°C for five days. Afterwards, the spore suspension (1x 10^6 spores mL^{-1}) was prepared following the protocol of Amobonye et al. (2021).

3.2.3 β-glucosidase production

B. bassiana SAN01 β-glucosidase production was conducted in 250 mL Erlenmeyer flasks containing 40 gL^{-1} Bambara haulm as a sole carbon source prepared in a 100 mL mineral salt solution (gL^{-1}); (0.7) $MgSO4.7H_2O$, (0.1) $FeSO_4$, (0. 3) K_2HPO_4 and (0.5) KH_2PO_4). The initial pH of the media was adjusted to 6.0 and autoclaved at 121°C for 20 min. The media was then inoculated with the spore suspension (1 mL) and incubated at 30°C for 9 days at 150 rpm.

Subsequently, the culture broth was filtered using a sterile muslin cloth and centrifuged at 10 000 x g for 15 minutes at 4°C. The supernatant obtained was used as the crude enzyme for subsequent experiments.

3.2.4 β-glucosidase assay and protein estimation

β-D-glucopyranoside (pNPG) was used as the chromogenic substrate for the quantification of β-glucosidase activity. The reaction mixture was made up of 0.9 mL of pNPG (5 mM) and 0.1 mL of the appropriately diluted enzyme, which was incubated at 35°C for 30 min. The reaction was terminated by adding 1 mL of 0.5 M Na_2CO_3 and the absorbance was measured at 410 nm using a spectrophotometer (Shimadzu UV- 1900i). One unit of β-glucosidase activity was defined as the amount of enzyme that generated 1 μmol of p-nitrophenol in 1 min at 35°C (da Costa et al., 2018). The protein content of the samples was determined by a modified Lowry method using bovine serum albumin as the standard (Hartree, 1972).

3.2.5 β-glucosidase purification

The crude β-glucosidase was purified to homogeneity by ammonium sulphate precipitation, ultrafiltration, and gel filtration chromatography. Firstly, the crude enzyme preparation was fractionated by ammonium sulphate precipitation (60-90%). The precipitates obtained after centrifugation at 7000 x g for 20 min were dissolved in a minimal volume of 0.1 M sodium acetate buffer (pH 5.0) and dialyzed against the same buffer for 24 h at 4°C. Subsequently, the dialyzed enzyme was concentrated using a 10 kDa Amicon cut-off filter. The concentrated fraction was applied (0.5 mL) onto a Superdex 200 10/300 GL column (1.0 cm×30 cm), equilibrated in 20 mM sodium acetate buffer (pH 5.0) and eluted with the same buffer at 0.5 mL min^{-1} (da Costa et al., 2018) in an AKTA protein purification system (GE Healthcare Life Sciences). Subsequently, fractions were collected and assayed for BGL activity and protein content.

3.2.6 SDS-PAGE and Zymogram

The molecular weight of the purified β-glucosidase was estimated using 12% cross-linked polyacrylamide gel and 4% stacking gel. Enzyme aliquots of 10 μg were loaded into the sample wells and electrophoresed at a constant voltage of 100 volts for 2 h at room temperature. After electrophoresis, the gel was stained with silver staining (González-Pombo et al., 2008). Subsequently, zymography was conducted using a native gel electrophoresis with 7% polyacrylamide gel and 4% stacking gel. After electrophoresis, the gel was immersed in 4-methylumbelliferyl-β-D-glucopyranoside prepared in 0.1 mM sodium acetate buffer, pH 5.0

at 45°C for 30 min in the dark. The release of methylumbelliferone from the substrate was observed under UV at 310 nm (Kuo et al., 2018).

3.2.7 Biochemical characterisation of β-glucosidase

3.2.7.1 pH optima and pH stability

To determine the optimum pH for β-glucosidase, different buffer preparations including acetate (pH 3.0-5.0), phosphate (pH 6.0- 7.0), and Tris-HCl (pH 8.0-9.0) at 0.05 M were used. The enzyme was pre-incubated in the respective buffer for 30 to 300 min at 35°C for the stability test and the residual activities were measured accordingly (Bonfá et al., 2018).

3.2.7.2 Temperature optima and thermostability

The optimum temperature for β-glucosidase was evaluated by incubating the reaction mixtures between 25 and 70°C at 5°C intervals. Subsequently, the thermostability of β-glucosidase was evaluated by pre-incubating the enzyme between 30 and 50°C for 300 min; samples were taken at 60 min intervals (Huang et al., 2021). Relative activity was measured according to standard enzyme assay.

3.2. 8 Hydrolysis of cellobiose by *B. bassiana* SAN01 β-glucosidase

B. bassiana SAN01 BGL was partially purified and concentrated via $(NH_4)_2SO_4$ precipitation (Gautério et al., 2021) and utilized in the hydrolysis of cellobiose. The 20 mL reaction mixture contained cellobiose at a final concentration of 1700 µg/mL and 50 U/mL of the *B. bassiana* BGL in 0.1 M sodium acetate buffer (pH 5.0) (Yepes et al., 2022). The reaction was performed for 24 h at 35°C, samples were withdrawn at 6 h intervals and the reaction was terminated by heating for 5 min at 100°C in a boiling water bath. The supernatant was collected by centrifugation for 10 min at 10,000×g and subsequently subjected to Thin-layer chromatography (TLC) and High-performance liquid chromatography (HPLC) to confirm the hydrolysis of cellobiose. TLC was done on silica gel TLC plates (60F254, Merk Co) using a solvent system made up of n-butanol, acetic acid, and water (2:1:1 v/v), the plates were dried for 5 min at 121°C and developed using orcinol reagent prepared in 10% sulfuric acid (Kim et al., 2018). Furthermore, the concentration of unhydrolyzed cellobiose and that of the released glucose was examined via HPLC using an Aminex HPX-87H BIO-RAD column ran with acetonitrile and water (1:2 v/v) at a flow rate of 0.7 mL/min. Elution was monitored using a RID detector (RID-10, Shimadzu) (Pei et al., 2012).

3.2.9. *In silico* structural characterisation of *B. bassiana* SAN01 β-glucosidase

3.2.9.1 Sequence retrieval and primary analysis

The sequence of *B. bassiana* SAN01 BGL was selected from the NCBI database (Accession no: KAH8714014.1) based on the estimated molecular weight of the purified enzyme in this study. The ProtParam tool was used to compute the aliphatic index, isoelectric point, the total number of negatively and positively charged residues, the instability index, and the grand average of hydropathicity. Subsequently, PSIPRED server http://bioinf.cs.ucl.ac.uk/psipred/), and Pfam search (http://pfam.xfam.org/) were employed in predicting the secondary structure and domains present in the BGL, respectively (Amobonye et al., 2021).

3.2.9.2 Homology modelling and molecular docking

The 3D structure of *B. bassiana* SAN01 BGL was modelled using ExPASy Swiss-Model and the quality of the predicted structure was evaluated by the Ramachandran plot which highlighted the energetically allowed regions (Amobonye et al., 2022).

3.2.9.3 Molecular docking

Initially, the active sites of the BGL were predicted using CASTp and MetaPocket 2.0 [21, 22]. Subsequently, Autodock 4.2 software was used for docking the modelled 3D structure of *B. bassiana* SAN01 BGL as the target and its two substrates, cellobiose, and p-nitrophenyl β-D-glucopyranoside, as the ligands. The structures of the ligands -cellobiose (PubChem CID: 294), and p-nitrophenyl β-D-glucopyranoside (PubChem CID: 92930) were obtained from the PubChem database (http://pubchem.ncb.nlm.nih.gov/) in SDF format and converted into PDB format using BIOVA Discovery Studio Visualizer (BIOVA, CA, USA). Polar hydrogens were added to the target molecule using Autodock tools, and a 40 × 40 × 40 grid box was used in the configuration file of Autodock Vina with the box centred at X: 32.128, Y: 26.721, Z: 27.453 coordinates. The receptor atom positions were fixed, and the torsion angle of the ligand glycosidic bonds was rotated to attain favourable docking. All other docking parameters were set to default. Subsequently, the most suitable BGL-ligand pose was chosen and visualised using PyMol (Zada et al., 2021a).

3.2.10. Statistical analyses

Statistical variance (ANOVA) was calculated with GraphPad Prism software (version 10). Differences between samples were undertaken to be statistically significant if $p < 0.05$.

3.3 Results and discussion

3.3.1 Purification of *B. bassiana* SAN01 β-glucosidase

To further purify *B. bassiana* SAN01 BGL after ammonium sulphate precipitation and dialysis, Gel filtration chromatography was used. Enzyme assay showed that BGL activity occurred between fractions B1 to B15 as shown by the peaks in figure 3.1. However, B9 was observed to contain purified BGL with a total protein of 2.99 mg and specific activity of 496 U/mg (Table 3.1), which was higher than some previously reported BGL such as those from *A. niger* (Narasimha et al., 2016), and *Penicillium citrinum* UFV1 (da Costa et al., 2018) which had specific activities of 60.6, and 349.2 U/mg, respectively.

The analysis of the protein pattern of the purified *B. bassiana* BGL revealed a single band (Figure 3.3. a) with an estimated size of 116 kDa using SDS-PAGE. The size of the protein was also confirmed via zymography, which showed the band at a similar position as observed on SDS-PAGE (Fig. 3.2. b). In the zymogram, it was observed that BGL hydrolyzed 4-methylumbelliferyl-β-D-glucopyranoside to release methylumbelliferone which fluoresced under UV light. The molecular mass obtained for *B. bassiana* SAN01 BGL was close to those recorded for the same enzyme from some *Aspergillus* spp. such as *A. versicolor* and *A. fumigatus* JCM 10253 with molecular mass 100 kDa (Huang *et al*., 2021) and 125 kDa (Saroj and Narasimhulu, 2022) respectively. However, BGL with a similar molecular mass was also obtained by Pal *et al*. (2010) from *Termitomyces clypeatus,* another filamentous fungus.

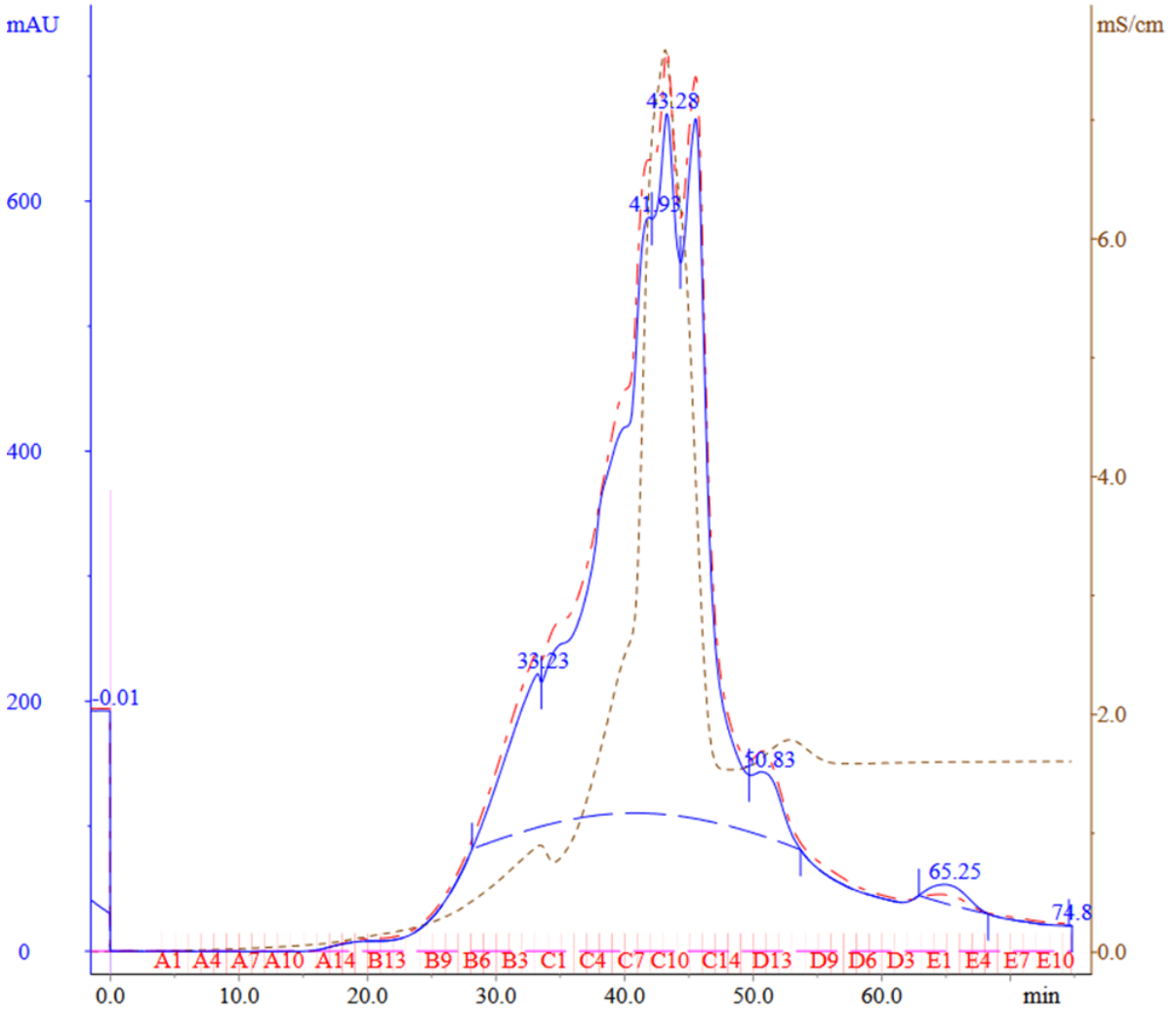

Figure 3.1:Elution profile of protein fractions from BGL of *Beauveria bassiana* on Superdex GL 10/300 gel filtration column

Table 3.1: Purification table of *B. bassiana* SAN01 β-glucosidase

Purification steps	Total activity (U)[a]	Total protein (mg)[b]	Specific activity	Recovery (%)
Crude enzyme	14886 ± 483	383 ± 18.7	38.9	100
$NH_4(SO_4)^2$ precipitation	5532 ± 104	73 ± 2.7	75.8	37.2
Ultrafiltration	2685 ± 61	28 ± 1.3	95.9	18
Gel filtration chromatography	1482 ± 26	2.99 ± 0.07	496	10

a= Crude extract of *B. bassiana* SAN01 after submerged fermentation in a total volume of 100 mL

b= Total protein content measured by Lowry-Hartree assay using BSA as the standards

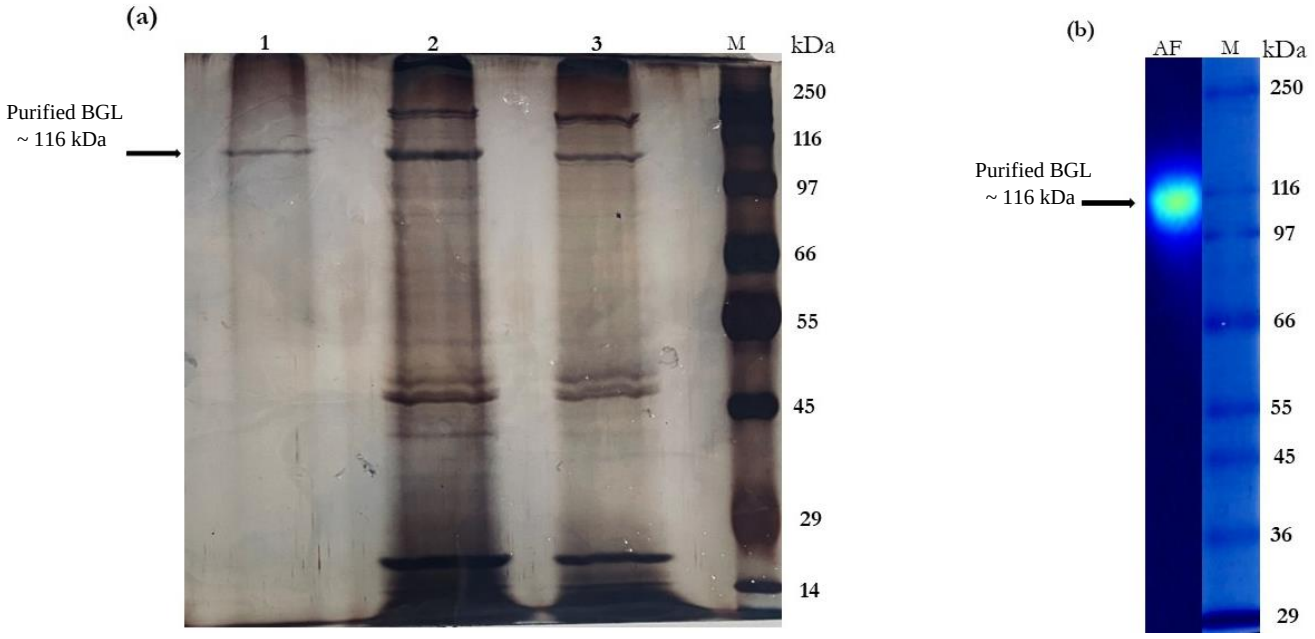

Figure 3.2:.(a). *B. bassiana* SAN01 β-glucosidase on 12% SDS-PAGE- Lane 1: Purified enzyme, Lane 2: Ammonium sulphate fraction, Lane 3: Crude enzyme, M: wide range protein marker (14- 250 kDa). (b) *B. bassiana* β-glucosidase activity on 7% Native PAGE gel containing 50 mM 4-methylumbelliferyl-β-D-glucopyranoside -AF: Active purified fraction, M: wide range protein marker (14- 250 kDa)

3.3.2 Characterisation of *B. bassiana* SAN01 β -glucosidase

3.3.2.1 Effect of pH on *B. bassiana* SAN01 β-glucosidase

The optimum pH for β-glucosidase was observed to be at pH 5.0 while substantial enzyme activity (83%) was also observed at pH 6.0. However, a further increase in pH led to a decline in the activity suggesting that the BGL from *B. bassiana* SAN01 is an acidophilic enzyme (Fig.3.3a). Concurring with these results, unpurified BGL from a different *B. bassiana* strain

was previously shown to be active under acidic conditions (Borgi *et al*., 2016); in addition, most fungal BGLs have been reported to be active at pH 4.0-6.0. Recent examples include the BGL from *Aspergillus fumigatus* (Saroj and Narasimhulu, 2022) and *Neofusicoccum parvum* (Singh et al., 2023b). The BGL in this study retained >60 residual activity after 4 h incubation at pH 4.0, 5.0, and 6.0, respectively (Figure 3.3b). It was also observed that the enzyme retained more than 80% of its initial activity at its optimum pH of 5.0 after 4 h of incubation while also retaining more than 65% of activity at pHs 4.0 and 6.0. Thus *B. bassiana* SAN01 BGL demonstrates a wide range of stability at acidic pH, which makes it suitable for use in industrial bioprocesses that require acidic conditions. However, at pH values above 7.0, the enzyme activity was observed to drop over time.

3.3.2.2 Effect of temperature on *B. bassiana* SAN01 β-glucosidase

B. bassiana SAN01 BGL displayed maximal BGL activity at 60℃, amongst the different temperatures evaluated, which is typical of a thermophilic microorganism. Borgi *et al.* (2016) had previously recorded an optimal temperature of 55℃ for crude BGL preparation from another *B. bassiana* strain. Similarly, other mesophilic fungi besides *B. bassiana* have been recorded to produce BGL with optimum activity between 40-60C°, such as *Aspergillus flavus* (Chen et al., 2019b), *A. fumigatus* JCM 10253 (Saroj and Narasimhulu, 2022), and *Aspergillus* sp. DHE7 (El-Ghonemy, 2021). Furthermore, it was observed that the *B. bassiana* BGL retained >50% of its initial activity after 300 min of incubation between 30℃ and 50℃, however, its stability declined above 50℃. The enzyme was also observed to retain ~ 85% of its activity at 30℃ followed by ~ 75% at 40℃ (Fig. 3.3d). In this regard, *B. bassiana* SAN01 BGL could be suitable for applications carried out at mid-temperature conditions. However, *B. bassiana* SAN01 BGL exhibited higher stability profile when compared to recombinant BGL from *Alteromonas* sp. which exhibited a $t_{1/2}$ of 20 min at 40℃ (Zada et al., 2021a). In contrast, *Microbulbifer* sp. ALW1 BGL losses original activity within 120 min at temperature between 40-45℃ (Jiang et al., 2021).

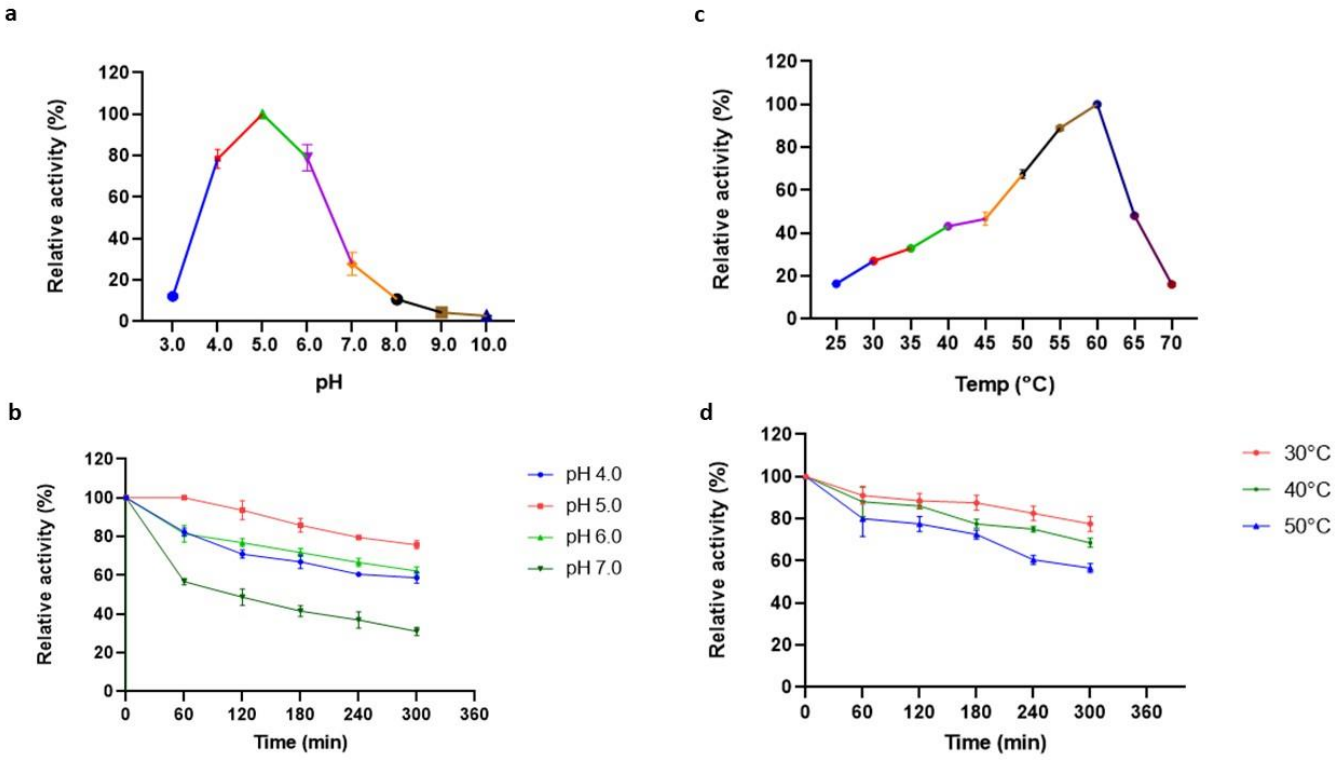

Figure 3.2:(a) pH optima (b) pH stability(c) temperature optima (d) thermostability of *B. bassiana* SAN01 BGL

3.3.2.3 Analysis of cellobiose hydrolysis by *B. bassiana* SAN01 β-glucosidase

The analysis of *B. bassiana* SAN01 BGL hydrolysis products by thin-layer chromatography (TLC) demonstrated the capability of the enzyme to transform cellobiose into glucose. Glucose intensity was observed to increase with reaction incubation time, while cellobiose intensity decreased with reaction time (Fig. 3.4a). The glucose released by the BGL overtime was further quantified and confirmed using HPLC. In this study, the absorption peak of glucose was observed at a retention time of 10.5 ± 0.4, which corresponded to the same retention time and peak area observed for the standard sugar used. Expectedly, the amount of glucose released from cellobiose was observed to increase with the incubation time, and close to 100% of glucose was obtained after 24 h of incubation. The complete hydrolysis was observed after 24 h in this study. However, in previous studies by Verma et al. (2013) and Pei et al. (2012), *A. niger* and *Thermoanaerobacterium thermosaccharolyticum* BGL yielded 77% and 100% glucose within 12 h and 6 h hydrolysis, respectively. Hence, in order to achieve increased efficiency and enhance its industrial applications, it is imperative to optimize other factors involved in the hydrolytic process of the *B. bassiana* BGL - such as the pH, enzyme loading, substrate loading and incubation temperature.

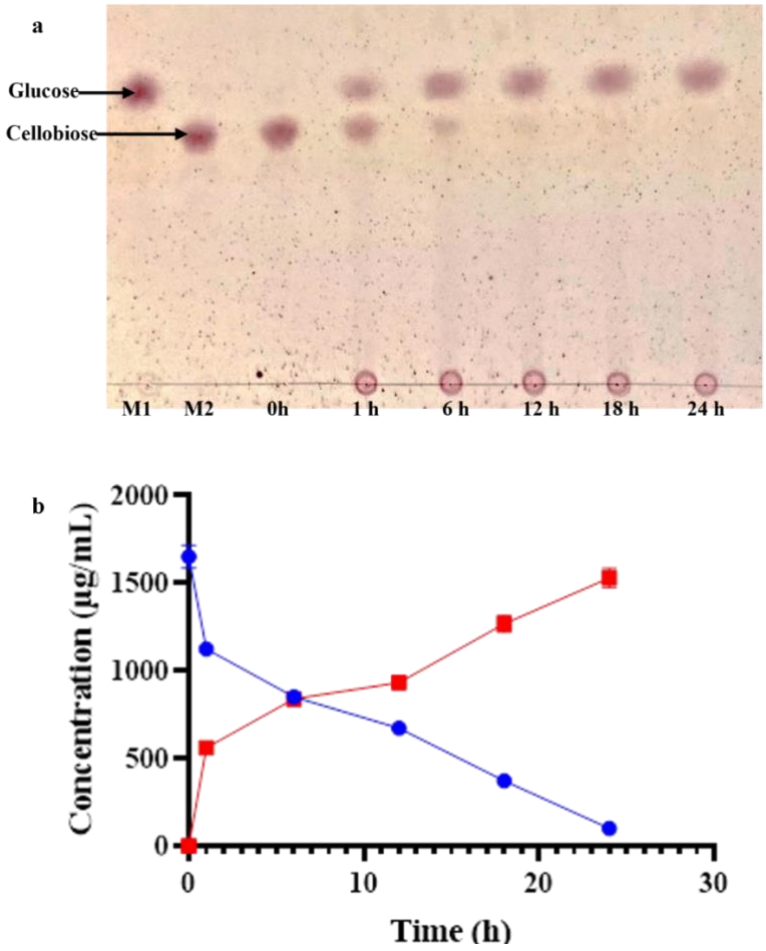

Figure 3.3. (a) Thin-layer chromatography analysis of cellobiose hydrolysis by *B. bassiana* SAN01 β-glucosidase, M1: Glucose standard M2: Cellobiose standard, Lanes 3, 4, 5, 6, 7, 8 show cellobiose hydrolysis after 0, 1, 6, 12, 18 and 24 h (b) Concentration of cellobiose of cellobiose hydrolysis (─) and glucose product (─) analysis as determined by HPLC

3.3.2.4 *In silico* structural prediction of *B. bassiana* SAN01 β-glucosidase

According to available literature, there is no information on the secondary and tertiary structure of any lignocellulolytic enzyme from *B. bassiana* SAN01 including BGLs, hence it is considered imperative to gain a preliminary insight into the structure of BGL using a computational approach. Subsequent to the estimation of the molecular weight of the purified BGL via SDS-PAGE, a sequence was selected from ~30 *B. bassiana* BGL sequences in the NCBI database for further analysis. The BGL sequence (Accession no: KAH8714014.1) was selected based on its approximated molecular weight of 116 kDa, which is close to the one obtained in this study. Analysis of the primary sequence showed that the enzyme comprises 1044 amino acids; in addition, the enzyme was predicted to have a molecular weight and

isoelectric point (pI) of ~ 116 kDa and 5.59, respectively. The enzyme was also observed to contain more negatively charged amino acid residues (aspartic acid and glutamic acid = 116) than positive residues (arginine and lysine = 97). The protein was also predicted to be quite stable judging from its computed instability index of 34.40, thus corroborating the results on the thermostability and pH stability obtained earlier in this study.

The *B. bassiana* BGL sequence was predicted with an aliphatic index (AI) and Grand average of hydropathicity (GRAVY) of 71.98 and -0.399, respectively. The AI has been used to predict protein thermostability based on the fraction of aliphatic amino acids in the protein structure, and higher AI values are synonymous with higher thermostability (Ikai, 1980, Kaur et al., 2020). Thus, the AI value of 71.78 obtained in this study, provides additional validation to notable thermostability obtained during characterisation studies. According to previous studies, negative GRAVY values may indicate the hydrophilicity of a protein (Bhagwat et al., 2021). Thus, from the results, *B. bassiana* SAN01 BGL can be predicted to be hydrophilic overall, raising the probability of its significant stability in an aqueous environment. It was also observed from domain analysis that *B. bassiana* SAN01 BGL belonged to the glycosyl hydrolases 1 family which also encompasses other accessory enzymes such as β-mannosidase, β-D-fucosidase, β-glucuronidase and 6-phospho- β-galactosidase.

For homology modelling of the *B. bassiana* SAN01 BGL, the alpha model structure of *B. bassiana* ARSEF 2860 (PDB ID: J5JSG7_BEAB2) was used as the template as it had the highest sequence identity (96.68%) and coverage (0.98) of all the suggested templates. The visualization of the model revealed that the generated model is built on the barrel type (β/α)8 architecture, forming coin slot-like, deep, and narrow active site (Fig. 3.5. a). This structure is conserved in the GH family1 and has been observed in glucosidases from *Phanerochaete chrysosporium* (Graebin et al., 2016) and *Coniophora puteana* (Zhou et al., 2023a). Furthermore, the Ramachandran plot of the *B. bassiana* BGL showed that 89.2% of its residues are in the most favoured regions and 8.9% residues in the additional allowed region (Fig. 3.5. b). The total distribution of these residues in the favoured region is being evaluated as 98.1%, which reflects acceptable model adequacy while an exceptionally low percentage of the residues were found in the outlier region (1.9%). Furthermore, the overall quality factor score predicted for the BGL model by ERRAT was 92.33 while VERIFY 3D also showed that 95.69% of the amino acid residues have an average 3D-ID score of >= 0.1, thus, pointing to the reliability of the generated model.

The docked complex of *B. bassiana* SAN01 BGL with the ligands showed the binding affinity scores of -6.2 and -7.2 (kcal mol^{-1}) for cellobiose and pNPG, respectively, thus, indicating that *B. bassiana* SAN01 BGL recognizes the artificial substrate pNPG more than the natural substrate, cellobiose. In accordance, a similar binding affinity score of -6.2 for cellobiose was obtained by Khairudin and Mazlan (2013) on *Paenobacillus polymyxa* BGL. The analysis of the pNPG-BGL complex revealed that the ligand interacted with the active site forming hydrogen bonds with the Lys 652, Lys 658, Arg 1000, and Arg 974 residues (Fig. 3.6. B). For the cellobiose-BGL complex, however, the hydrogen bond interactions were formed with Asp 658, Arg 974, and Ser 1001 (Fig 3.7. C). All the molecular interactions recorded in both enzyme-ligand complexes are presented in Table 3.2. These results indicate that *B. bassiana* SAN01 BGL binds significantly with both natural and synthetic substrates unlike the BGL from *Neosartorya fischeri* which showed no hydrogen bond interaction with cellobiose (Ramachandran et al., 2012). Hence, the BGL in this study can be classified into the third class of glucosidase which acts on both aryl- β-D-glucosides and disaccharides due to the broad substrate specificity.

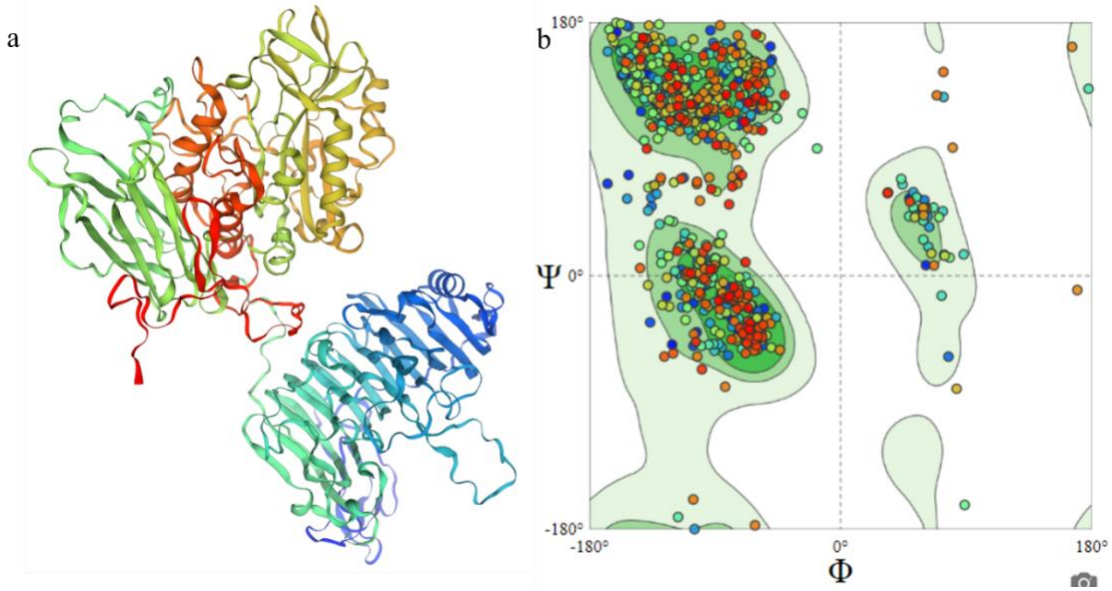

Figure 3.4:(a) 3D structure of *B. bassiana* SAN01 BGL (b) Ramachandran plot of *B. bassiana* SAN01 BGL; 92.71% favoured region, 0.86% outlier

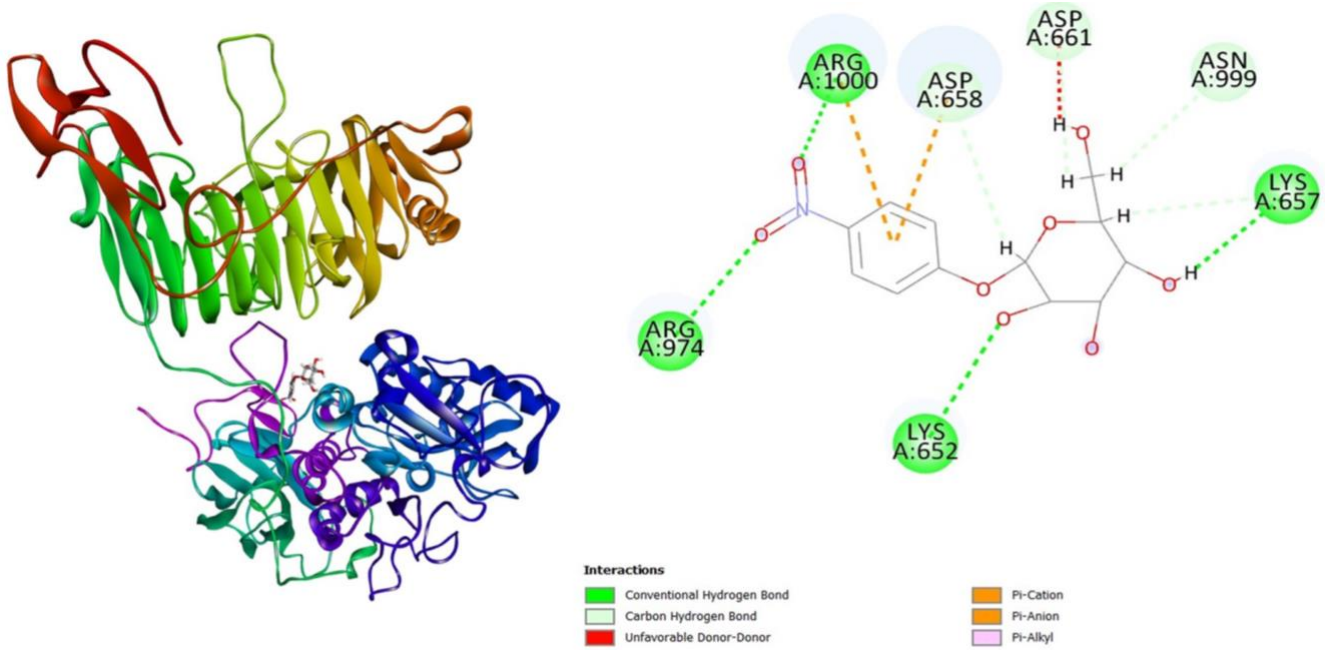

Figure 3.5:*B. bassiana* SAN01 BGL-pNPG complex and molecular interactions between pNPG and ligand sites of *B. bassiana* SAN01 BGL

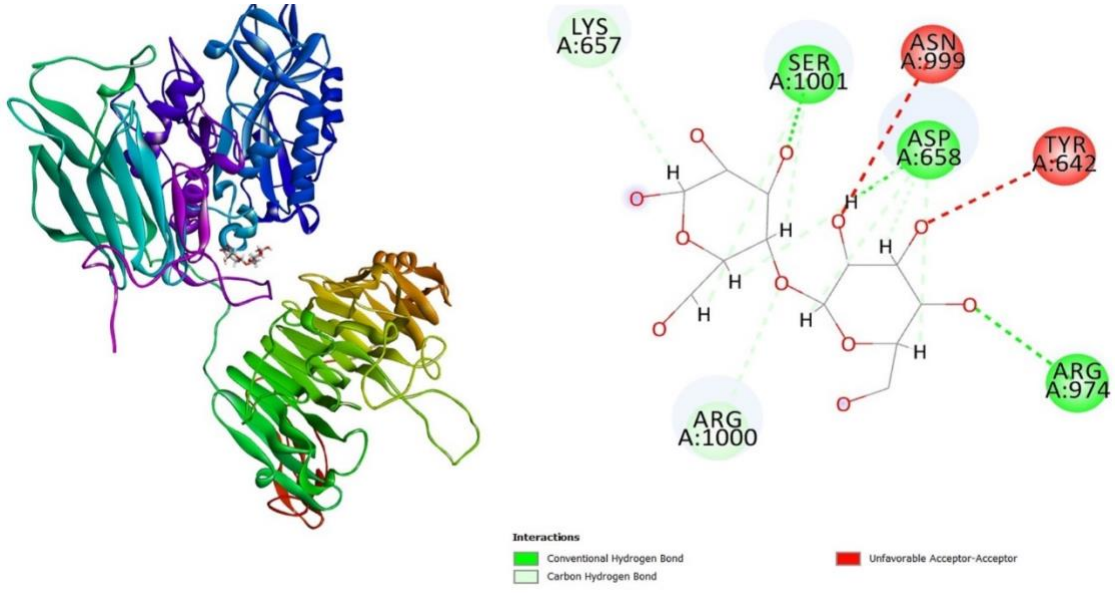

Figure 3.6: *B. bassiana* SAN01 BGL-cellobiose complex and interactions between cellobiose and ligand sites of *B. bassiana* SAN01 BGL

Table 3.2: Active site interaction in *B. bassiana* SAN01 BGL-substrate complexes

Substrate	Interactive bonds between enzyme and ligands	Active site amino acids
pNPG	Hydrogen	Lys 652, Lys 658, Arg 1000, Arg 974
	Carbon-hydrogen	Asp658, Asp 661, Asn 999
	Pi cation, Pi anion	Arg 1000, Asp 658
	Adverse acceptor–acceptor	Asp 661
	Hydrogen	Ser1001, Asp 658, Arg 974
Cellobiose	Carbon-hydrogen	Lys 657, Arg 100
	Adverse acceptor-acceptor	Asn 999, Tyr 642

3.4 CONCLUSION

In this study β-glucosidase from a fungal endophyte, *B. bassiana* SAN01 was purified to homogeneity. This particular β-glucosidase exhibited several noteworthy characteristics, including stability at acidic pH levels. Additionally, molecular studies indicate that *B. bassiana* SAN01 BGL exhibits activity towards both aryl-β-D-glycosides and disaccharides. Consequently, these findings imply that *B. bassiana* SAN01 BGL can be classified as a class III BGL, owing to its wider range of substrate specificity. The *B. bassiana* SAN01 BGL successfully released glucose from cellobiose. This characteristic makes it a promising candidate for various biotechnological applications, such as bioethanol production from lignocellulosic materials, food processing, and the biosynthesis of aryl glycosides in the pharmaceutical industry. However, additional investigation is necessary to further examine its potential applicability, particularly when used in conjunction with other cellulase enzymes, to develop more effective enzyme cocktails for improved saccharification of lignocellulosic biomass.

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVES

The global β-glucosidases (BGLs) market is currently estimated at ~400 million USD, and it is expected to double in the coming years, a trend that is mainly ascribed to the demand for the enzyme for use in different biotechnological processes including biofuel processing, food processing, feed processing, pulp and paper processing and pharmaceutical production. However, most microbial BGLs are inhibited by their end product, glucose, and stable at mid-range conditions, thus creating a bottleneck in the utilisation of these enzymes in various biotechnological applications. To circumvent this bottleneck, there is a need to look for new strains that synthesis BGL with robust properties including, glucose tolerant, cellobiose specificity, and stable at wider range pH conditions as well as higher temperature respectively. This could be achieved by isolating BGLs from high-yielding varieties of microorganisms. In this regard, the present study was focused on the production of BGL by a locally isolated *Beauveria bassiana* SAN01, followed by the optimisation of its BGL production using response surface methodology. Moreover, the enzyme was purified, characterised, and subsequently utilised for cellobiose hydrolysis. In this chapter, highlights of the significant findings of the study are summarised.

The *B. bassiana* SAN01 strain was found to proliferate on different agricultural residues (Bambara, corn cob, molasses bran, wheat bran, and wheat straw) utilised in this study, while producing significant quantities of BGL under submerged fermentation. Interestingly, amongst the biomass tested, Bambara haulm - an underutilised African legume biomass-supported the highest BGL production (132.71 U/mL), followed by the commonly used biomass, wheat bran (87.80 U/mL). The presence of cellulose and hemicelluloses, which are potent inducers of lignocellulose degrading enzymes in Bambara and wheat bran, might haave aided in the high level of BGL secretion observed with these two biomasses. Subsequently, for maximal production of the *B. bassiana* BGL, optimisation of the enzyme production process was carried out via response surface methodology using the best-performing biomass (Bambara haulm) as the sole carbon source. The two-step optimisation which included Plackett Burman design and Central Composite Design, resulted in a ~5.32-fold increase from the unoptimised production level of 132.71 U/mL to 711.0 U/mL. Bambara - 57 g/L, KCl - 302 mg/L, NaCl - 154 mg/L, agitation -150 rpm, and incubation time - 223 h were identified as the optimal

conditions for maximum production of BGL. The optimised BGL production level recorded in this study is one of the highest-ever reported for BGL from any endophytic fungus.

Further in this study, the enzyme was purified to homogeneity using ammonium sulphate precipitation followed by gel filtration chromatography. At the end of the purification process, yield recovery and specific activity of 10% and 496 U/mg respectively were recorded for the enzyme. The molecular weight of the purified enzyme was estimated to be 116 kDa by SDS-PAGE and zymogram. This is the first study to purify BGL from any of strain of *Beauveria* to homogeneity. The biochemical characterisation of the BGL revealed that it exhibits maximum activity at pH 5.0 and 60°C, therefore, *B. bassiana* SAN01 BGL can be classified as mildly acidophilic and thermotolerant. The pH stability analysis of the BGL showed more than 80% of its initial activity at pH 5.0 after 4 h of incubation, while also retaining more than 65% of initial activity at pHs 4.0 and 6.0 which suggests its suitability in industrial bioprocesses that require acidic conditions. The enzyme also displayed significant thermal stability from temperatures 30-50°C, retaining almost 60% of its activity at 50°C after 4 h of incubation.

The saccharification potential of *B. bassiana* BGL on cellobiose was qualitatively analysed by TLC and subsequently quantified via HPLC. Chromatography results revealed that the enzyme efficiently hydrolysed cellobiose into glucose, as glucose intensity on TLC was observed to increase with the incubation time, while cellobiose intensity decreased with reaction time, indicating hydrolysis. Furthermore, close to 100% of glucose was obtained after 24 h of incubation., however, it is imperative to optimize the hydrolytic process in order to achieve maximal saccharification.

The study was concluded by the structural characterisation of the enzyme using *in silico* methods which included the molecular docking of the enzyme with two of its common substrates, *viz*., cellobiose and 4-nitrophenyl-β-D-glucopyranoside. This was necessitated by the fact that there is no information on the 3D structure of *B. bassiana* SAN01 BGL(s). However, the sequence of *B. bassiana* SAN01 BGL was selected from the NCBI database (Accession no: KAH8714014.1) based on the estimated molecular weight (~116 kDa) of the purified BGL which was established earlier in this study. From the *in silico* analysis, it was predicted that the enzyme comprises 1044 amino acids, and is hydrophilic in nature, judging from its computed instability index of 34.40. The generated 3D model of *B. bassiana* BGL was observed to be built on the $(\beta/\alpha)_8$-barrel architecture, which is typical of the glycosyl hydrolase family1. The presence of 92.7% of the amino acid residues of *B. bassiana* SAN01 BGL in the Ramachandran favoured region confirmed the reliability of the generated model. The

molecular studies indicate that *B. bassiana* SAN01 BGL exhibits activity towards both aryl-β-D-glycosides and disaccharides with the binding affinity energies of -7.2 and -6.2 (kcal mol^{-1}) for 4-nitrophenyl β-D-glucopyranoside and cellobiose respectively with Lys-657, Asp-658, and Arg-1000 predicted as the core amino acid residues in the enzyme catalytic site. Consequently, these findings imply that *B. bassiana* SAN01 BGL can be classified as a class III BGL, owing to its wider range of substrate specificity.

In summary, this study has revealed a novel BGL with the highest ever production levels recorded for these enzyme from local strain of the entomopathogenic fungal endophyte, *B. bassiana* SAN01, with a remarkable acidic pH stability and significant potential in cellobiose degradation, thus providing a new carbohydrase for biotechnological process revolving around cellobiose hydrolysis as well as acidic conditions respectively. However, there is still a need for further investigation on the heterologous production of *B. bassiana* SAN01 BGL to satisfy industrial requirements such as higher product yield, efficient product recovery, and reduced cost of purification. Furthermore, the upscaled production of this enzyme at the bioreactor level can also be explored to increase its economy of scale. In addition, the utilisation of biophysical techniques such as circular dichroism, X-ray crystallography, fluorescence spectroscopy, and cryogenic electron microscopy will be instrumental in fully understanding and establishing the structural-functional relationship of this enzyme while also validating or disproving the *in silico* properties obtained in this study.

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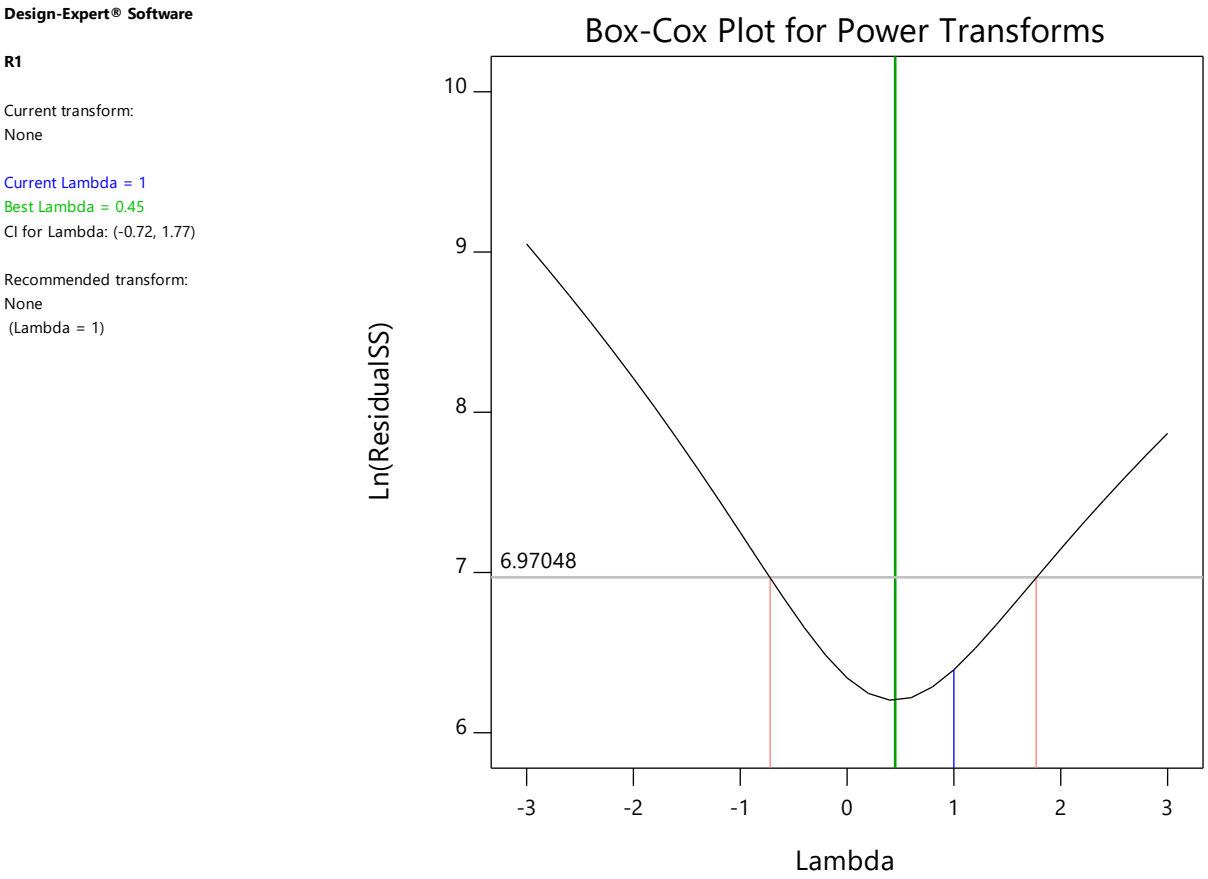
APPENDICES

Appendix 1

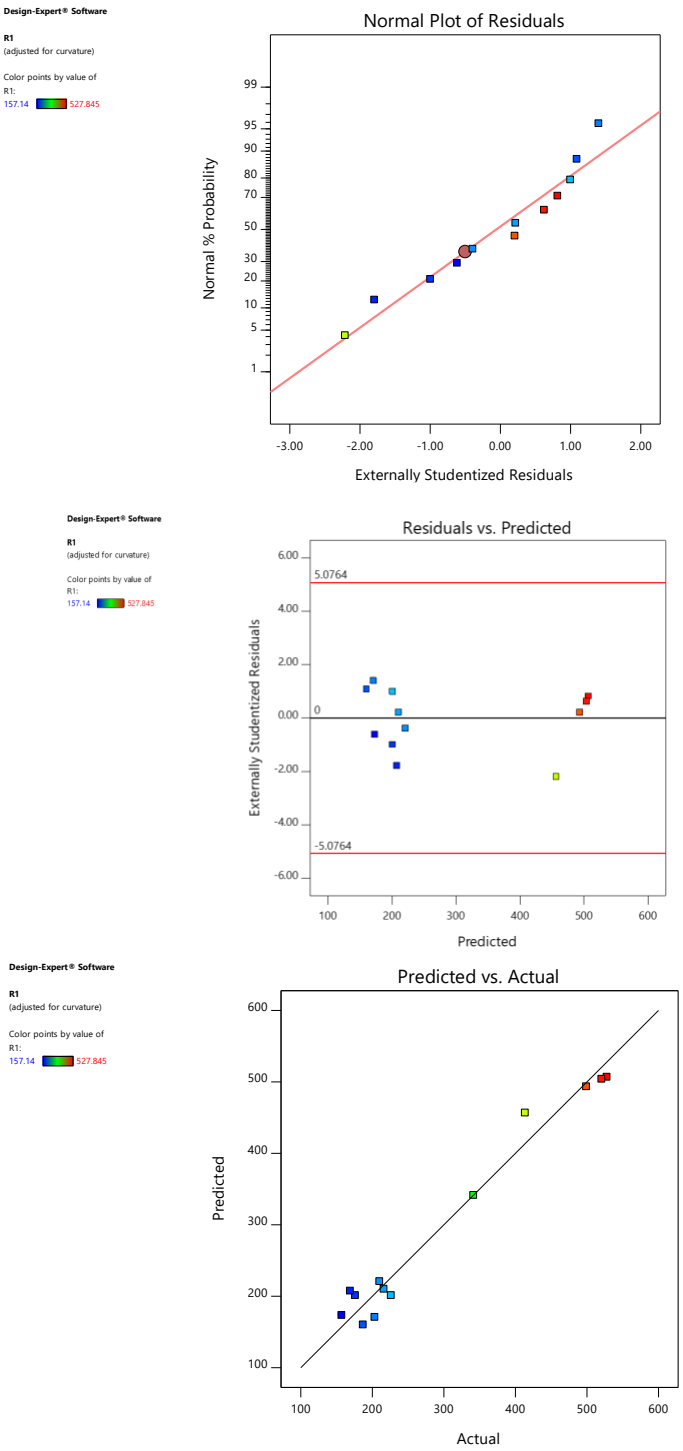
Coefficient estimates for β-glucosidase Plackett Burman model

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	292.19	1	10.24	267.15	317.24	
A-Bambara	101.72	1	10.24	76.67	126.77	1.0000
D-KCl	-46.57	1	10.24	-71.61	-21.52	1.0000
F-NaCl	-41.29	1	10.24	-66.33	-16.24	1.0000
J-Shaking	39.92	1	10.24	14.88	64.97	1.0000
L-Incubation time	64.95	1	10.24	39.90	89.99	1.0000
Ctr Pt 1	49.18	1	36.91			



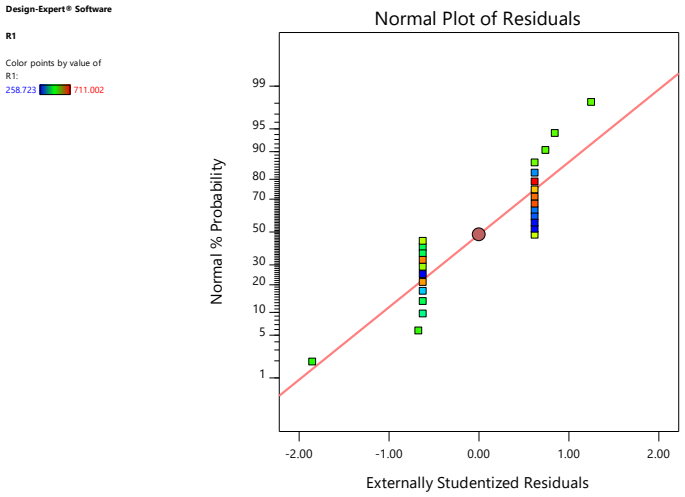
Appendix 2

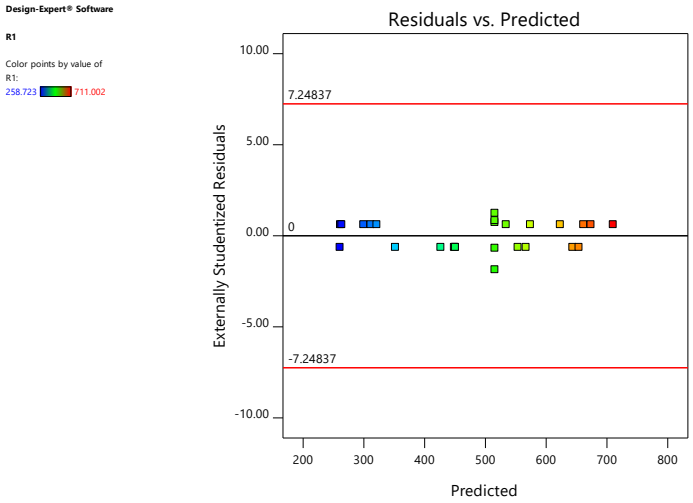
Box-Clot plot for power transform of β-glucosidase production

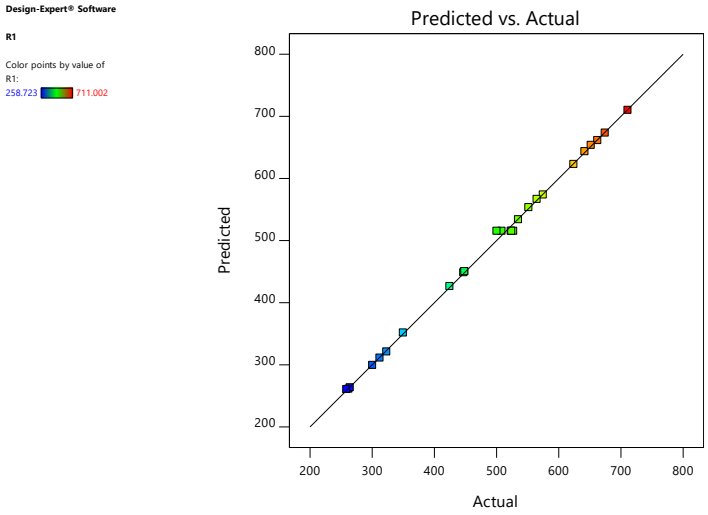


Appendix 3

Distributive plotting of the experimental data versus predicted values for β-glucosidase production

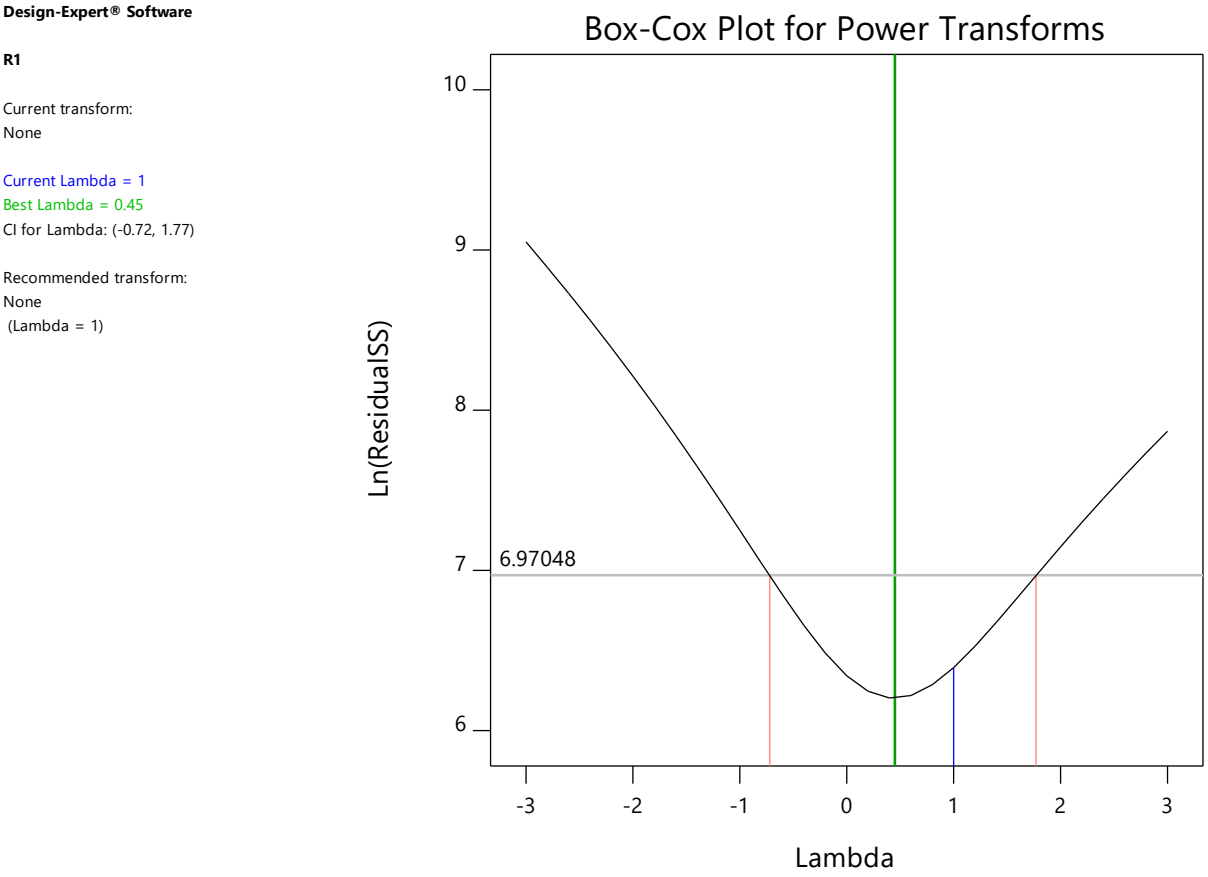






Appendix 4

Coefficient estimates for β-glucosidase Central composite design mode



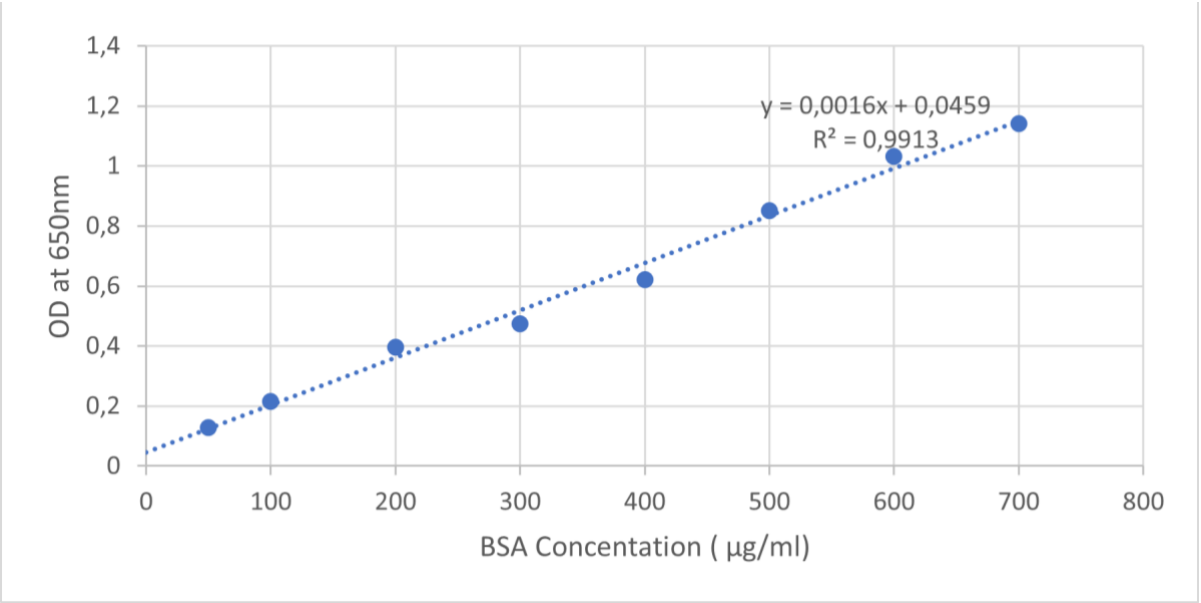
Appendix 5

Box-Clot plot for power transform of β-glucosidase production by CCD

Appendix 6

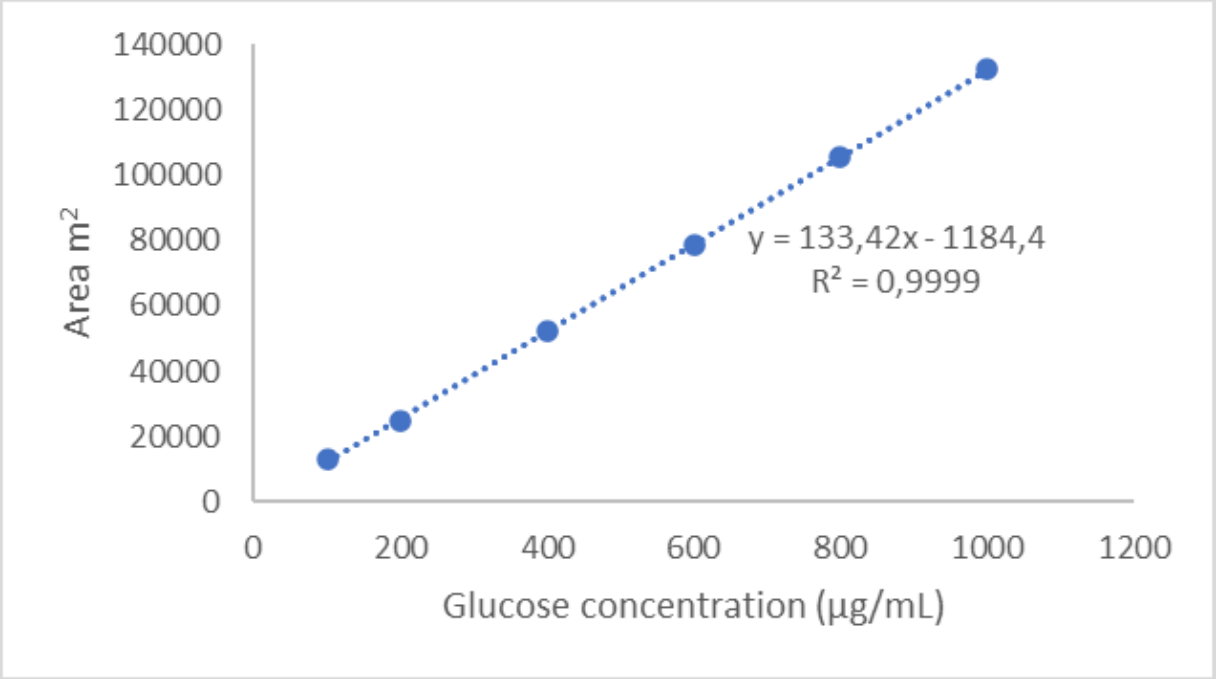
Coefficient estimates for β-glucosidase CCD model

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	515.41	1	4.54	503.73	527.09	
A-Bambara	105.15	1	4.25	94.24	116.06	2.65
B-KCl	-28.33	1	4.25	-39.24	-17.41	2.65
C-NaCl	-32.41	1	4.25	-43.33	-21.50	2.65
D-Shaking	-55.82	1	4.25	-66.74	-44.91	2.65
E-Incubation time	20.53	1	4.25	9.62	31.44	2.65
AB	21.97	1	6.25	5.89	38.04	3.59
AC	6.94	1	6.25	-9.14	23.01	3.59
AD	15.06	1	6.25	-1.01	31.14	3.59
AE	7.63	1	6.25	-8.45	23.70	3.59
BC	-17.80	1	6.25	-33.87	-1.72	3.59
BD	-9.61	1	6.25	-25.69	6.47	3.59
BE	-39.96	1	6.25	-56.04	-23.88	3.59
CD	-25.03	1	6.25	-41.11	-8.95	3.59
CE	-54.56	1	6.25	-70.64	-38.49	3.59
DE	-37.10	1	6.25	-53.18	-21.03	3.59
A^2	-19.12	1	2.40	-25.29	-12.94	1.02
B^2	-4.11	1	2.40	-10.28	2.07	1.02
C^2	-2.35	1	2.40	-8.52	3.83	1.02
D^2	11.03	1	2.40	4.86	17.20	1.02
E^2	-38.12	1	2.40	-44.29	-31.94	1.02



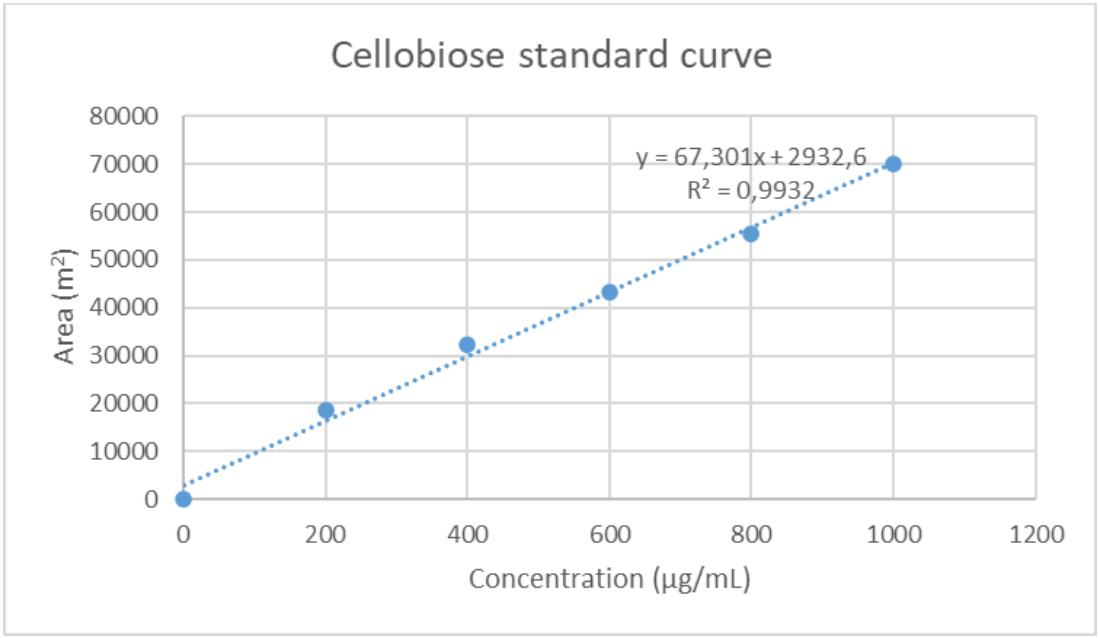
Appendix 7

BSA standard curve for protein assay



Appendix 8

Glucose standard curve for HPLC



Appendix 9

Cellobiose standard curve for HPLC