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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]

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

1 Introduction

Beta-glucosidases (E.C.3.2.1.21) or β-glucosidases or Bgls is a class of glycosyl hydrolases that catalyze the hydrolysis of β-glycosidic bonds in short-chain oligosaccharides and alkyl- and aryl β-D-glucosides. 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 [1]. The terminal reaction is catalyzed by Bgls which hydrolyze the cellobiose (released by the action of endoglucanase and exoglucanases) into glucose monomers [2]. Therefore, the efficient hydrolysis of cellulose, especially for the production of biofuels, is facilitated by the enzymatic actions of Bgls. In addition, the enzymes also possess some side activities that have been explored for other industrial applications [3]. 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, and the

✉ Santhosh Pillai
santhoshk@dut.ac.za

Buka Magwaza
bukawelcome4359@gmail.com

Ayodeji Amobonye
dejiamobonye@gmail.com; ayodejia1@dut.ac.za

Prashant Bhagwat
pkbhagwat9988@gmail.com; prashantb@dut.ac.za

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

production of alkyl glucosides that are useful in cosmetics and detergents production [4].

With the global shift towards cleaner energy production as highlighted in the UN Sustainable Developmental Goal (UN SDG) 7, Bgls have become prominent among the most sought-after enzymes. Consequently, Bgls 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 [5]. Bgls from several fungal genera such as *Aspergillus* [6], *Fusarium* [7], and *Trichoderma* [8] 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, which results in 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 [9, 10, and 11]. 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 [9] and wheat bran [12], respectively, as well as the actinomycetes *Jiangella alba* which utilized wheat straw for the same purpose [13]. 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. [10], 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) [11]. 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 [14].

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 [15]. The fungus is non-pathogenic to humans, animals, and plants [16]; 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 [17]. It was further observed during preliminary screening that the fungus' Bgl production level under unoptimized conditions was higher than the previously reported for any *Beauveria* strain or other entomopathogenic fungal endophyte.

Process parameter optimization 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 [18]. Historically, the optimization 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 labor-intensive, especially when dealing with a substantial number of independent variables [19]. However, the conventional optimization 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 optimization of fermentation processes [20]. This statistical approach decreases the overall number of required experiments and better elucidates the relationships between various elements and the fermentation output [21]. 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 [22].

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 Methodology

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), cellobiose, and glucose 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 optimization as well as for the validation of the generated model.

2.2 *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 5 days. Afterward, *B. bassiana* spore suspension (1×10^6 spores/mL) was prepared following the protocol of Amobonye et al. [23].

2.3 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) (NH4)$_2$SO$_4$ (4.0), KH$_2$PO$_4$ (1.0), MgSO$_4$.7H$_2$O (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 (1×10^6 spores/mL) and incubated for 8 days at 30 °C and 150 rpm. Afterward, the culture broth was filtered using a sterile muslin cloth and centrifuged for 15 min at 4 °C and $10,000 \times g$. The supernatant retrieved after centrifugation was used as the crude enzyme for subsequent experiments.

2.4 Enzyme assay

β-D-glucopyranoside (pNPG) was used as the chromogenic substrate for the quantification of *B. bassiana* SAN01 Bgl activity. The reaction mixture comprised 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$_2$CO$_3$ (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 35 °C [24]. The protein content of the samples was also

determined by a modified Lowry method using bovine serum albumin as the standard [25].

2.5 Statistical optimization of the fermentation conditions

2.5.1 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 [26]. 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 Eq. (1).

$$Y = \beta 0 + \Sigma \beta i X i \tag{1}$$

Y is the mean of Bgl activity; *β0* is the intercept of the model; *βi* is the linear coefficient; *Xi* is the the value of an independent component.

Optimization at this level involved a total of 11 components, consisting of nine production media components and two physical components, which were selected from preliminary studies and previous literature [27, 28]. The experimental design included 13 runs, with 1 center 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 summarized in Table 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 optimized in CCD.

Table 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$_4$)$_2$SO$_4$	(g/L)	B	2	4	6
KH$_2$PO$_4$	(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

2.6 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 ($-\beta$, -1, 0, 1, β) with Table 2 summarizing 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).

$$Y = \beta0 + \Sigma\beta iXi + \Sigma\beta iiXi^2 + \Sigma i\Sigma\beta ijXiXj \qquad (2)$$

Y is the mean of Bgl activity; *Xi* and *Xj* are the independent components; *β0* is the intercept constant, *βi* is the linear coefficients; *βii* is the quadratic coefficient; *βij* is the coefficient of two-component interaction. Each variable in the equation was fitted to determine its significance, and an ANOVA (analysis of variance) test was performed to observe the goodness of fit. The interactions among the evaluated components were represented and visualized in contour plots [29].

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 [30].

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

B. bassiana SAN01 Bgl was partially purified and concentrated via (NH$_4$)$_2$SO$_4$ precipitation [31] 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 mM sodium acetate buffer (pH 5.0) [32]. The reaction was performed for 24 h at 35 °C, samples were withdrawn at 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 cellobiose hydrolysis. TLC was done on silica gel plates (60F254, Merck 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 [33]. Furthermore, the concentrations of unhydrolyzed cellobiose and that of the released glucose were examined via HPLC using an Aminex HPX-87 H column (BIO-RAD) 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).

2.8 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 $p < 0.05$.

3 Results and discussion

3.1 Valorization of lignocellulosic biomass for β-glucosidase production

In this study, *B. bassiana* SAN01 was observed to proliferate remarkably on different lignocellulosic biomass and produced Bgl in high quantities. Amongst the biomass tested, Bambara haulm and wheat bran were the most promising, supporting higher β-glucosidase yields of 132.71 U/mL and 87.80 U/mL, respectively (Fig. 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 [34]. However, recent studies have shown that it contains a significant quantity of cellulose (37%) and hemicellulose (15%) [35]; hence, it possesses the potential for valorization, 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

Table 2 The range of significant components used in CCD

Variable	Symbol	$-\beta$	-1	0	$+1$	$+\beta$
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

Fig. 1 *B. bassiana* SAN01 β-glucosidase production using different lignocellulosic biomass as the sole carbon source (bars with the same letter superscripts are not significantly different at $p < 0.05$)
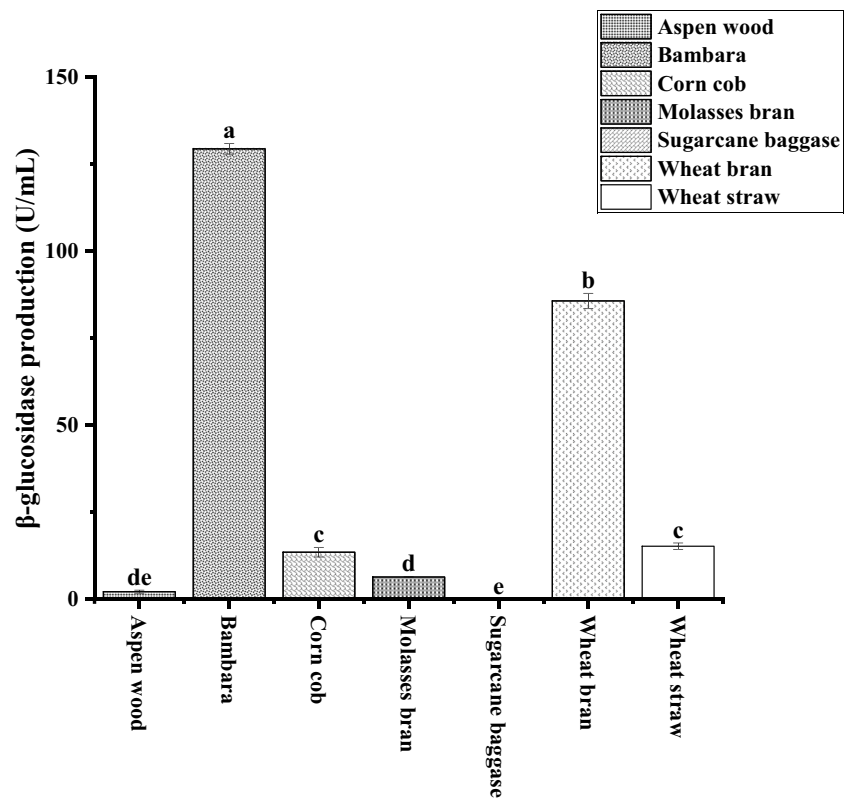
utilization of Bambara haulm as a fermentation substrate, we hypothesize that being a leguminous plant, it contains an adequate amount of required nutrients to support robust fungal metabolism and enzyme production. Furthermore, as observed in wheat bran and other lignocellulosic biomass, its constituent 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 [36, 37]. 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* [38]. 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 [39]; 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 unoptimized Bgl production level obtained from *B. bassiana* SAN01 in this study is 25.38-fold higher than the 5.20 U/mL from another strain of *Beauveria* [40]. Bambara haulm, being the best biomass for Bgl production by *B. bassiana* SAN01, was further used for optimization using RSM.

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 optimization are summarized in Table 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), 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 (Supplementary data). 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 were established as the most significant factors (Table 4). These results are in agreement with the previous studies that identified incubation time [19, 41] and agitation speed [38, 41] as significant factors in Bgl production by different

Table 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

Table 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	48,077.00	36.24	<0.0002
A-Bambara	1.242E+05	1	1.242E+05	98.75	<0.0001
D-KCl	26,019.86	1	26,019.86	20.69	0.0039
F-NaCl	20,453.61	1	20,453.61	16.27	0.0069
I-Agitation speed	19,128.06	1	19,128.06	15.21	0.0080
K-Incubation time	50,616.59	1	50,616.59	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

Fig. 2 The Pareto chart of standardized effects for the production of *Beauveria bassiana* SAN01 β-glucosidase (the orange and blue colors represent positive and negative effects, respectively)

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 [38]. 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 3). 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 speed (J) and incubation time (L).

Bgl production level = 292.19 + 101.72 A − 46.57D − 41.29 F + 39.92 J + 64.95 L.

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 optimization of the response and the curvature of *B. bassiana* Bgl yield.

3.3 Optimization 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 4. 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 optimization of fermentation conditions for enhanced *B. bassiana* SAN01 Bgl production (Fig. 3). The analysis of the coefficient estimates of the CCD revealed the positive linear effects of incubation time, Bambara concentration, and agitation speed and the negative effects of KCl and NaCl concentrations on Bgl production (Supplementary data). The observed experimental values vary from 258.7 to 711 U/mL (Table 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 6). Adeq Precision is a measure of the signal-to-noise ratio, with a value greater than 4 being desirable [22, 42]. 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 [22]. 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* is the concentration of Bambara, *B* is the KCl concentration, *C* is the NaCl concentration, *D* is the agitation speed, and *E* is the incubation time. Thus, the model quadratic regression is given as:

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

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

Table 5 Experimental design in terms of actual variables and response of the central composite design (CCD)

Run	Bambara haulm concentration (*AA*)	Incubation time (*E*)	Agitation speed (*D*)	KCl (*B*)	NaCl (*C*)	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

**Table 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	24,108.31	201.63	<0.0001	Significant
A-Bambara	73,340.67	1	73,340.67	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	20,671.54	1	20,671.54	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	30,109.61	1	30,109.61	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
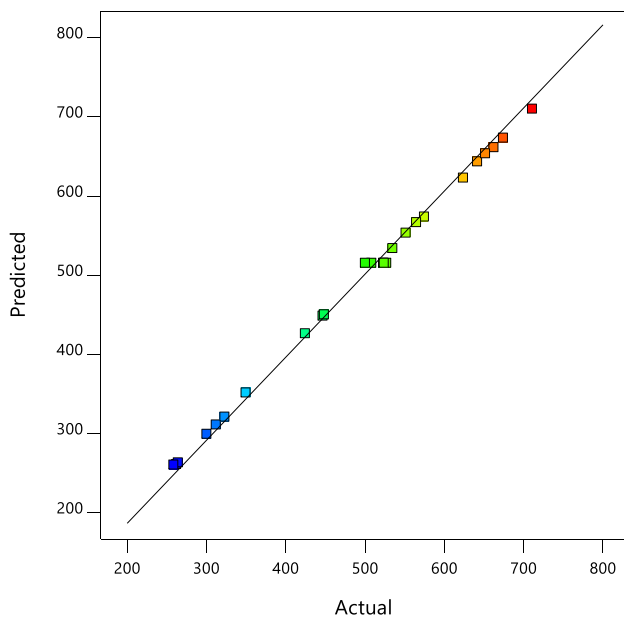
Fig. 3 Normal probability plot of predicted versus actual response of *Beauveria bassiana* SAN01 β-glucosidase activity

Lambda value of 1 for the response variable, hence, demonstrating that the response does not require any transformation for *B. bassiana* Bgl production (Supplementary data). Thus, three-dimensional response surface plots (Fig. 4) were generated based on the CCD quadratic regression to demonstrate the pair-wise interactive effects of two variables and their effect on *B. bassiana* 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. 4). 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 5. *B. bassiana* Bgl production was observed to increase with increasing concentration of Bambara and decreasing concentration of KCl (Fig. 4a). The interaction between NaCl and KCl as illustrated in Fig. 4b showed that Bgl production was favored 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
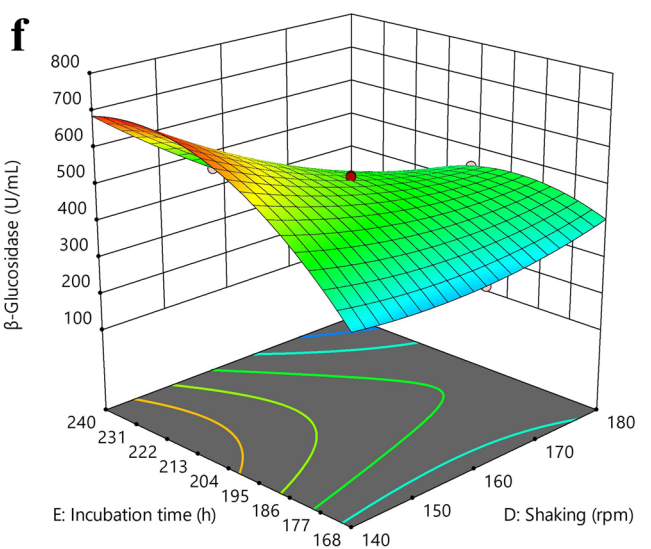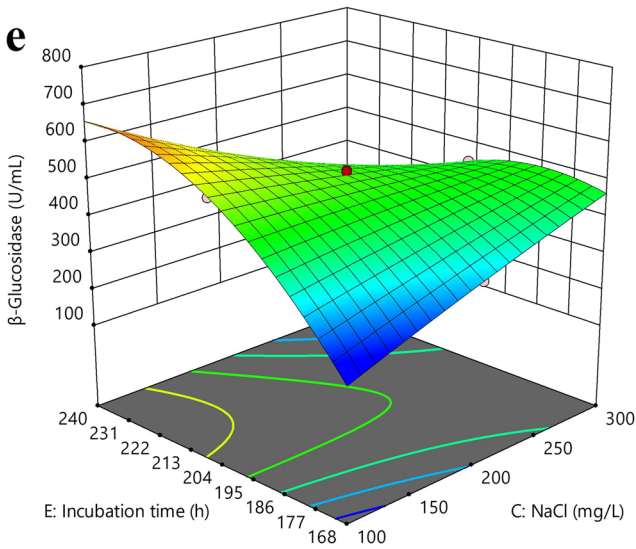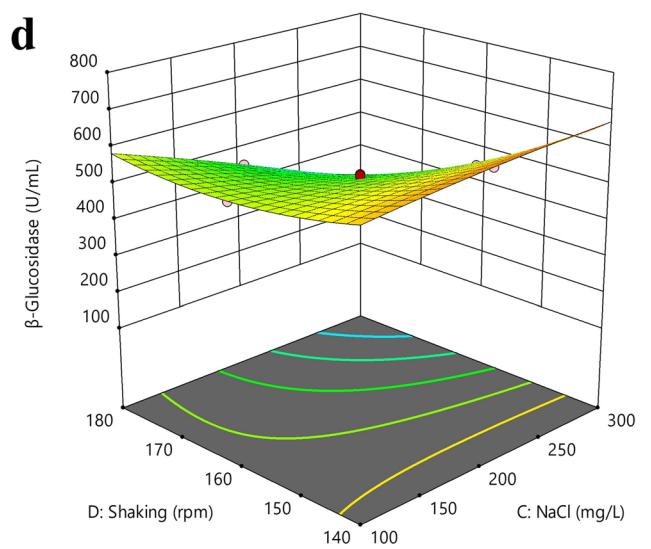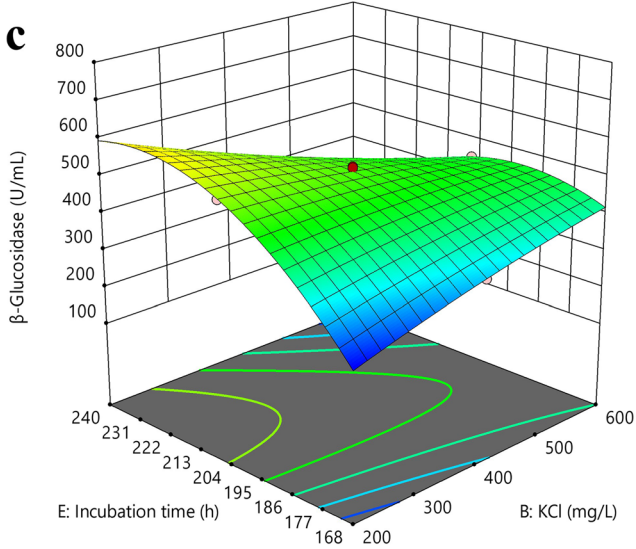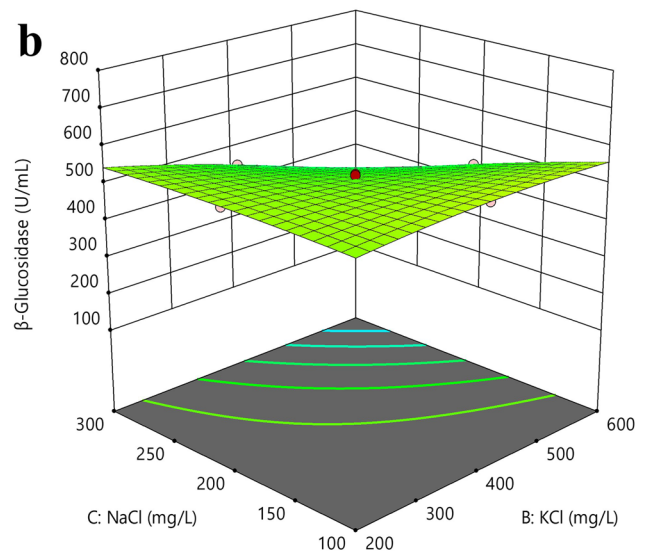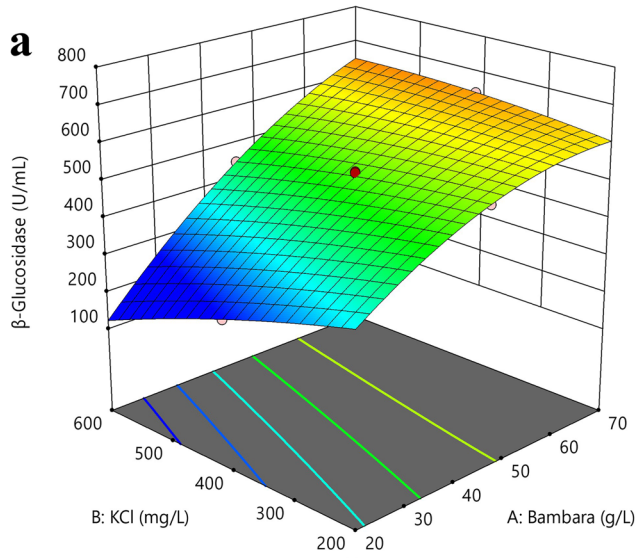
◄ **Fig. 4** 3D-response surface plots for the β-glucosidase activity: **a** effect of KCl versus Bambara concentration, **b** effect of KCl versus NaCl concentration, **c** effect of incubation time versus KCl concentration, **d** effect of agitation speed versus NaCl concentration, **e** effect of incubation time versus NaCl concentration, and **f** effect of incubation time versus agitation speed

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. 4c, e, and f). The decline in Bgl production after 223 h could be attributed to nutrient depletion which affected *B. bassiana* metabolism or the accumulation of proteases and other byproducts resulting in Bgl degradation as well as cell autolysis [43]. These results are corroborated by previous studies where enzyme production was also noted to have steeply declined beyond the optimum incubation time [19–21]. Interactions between incubation time and NaCl (CE) were more statistically significant amongst all other tested interactions in this study with the observed *p* value of 0.0003 (<0.05) (Fig. 4e). In addition, from the contour plots graphs, agitation speed between 140 and 150 rpm and NaCl concentration between 150 and 200 (mg/L) resulted in higher Bgl production (Fig. 4d). 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 optimization of medium components and physical components via statistical methods [38, 41].

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 these 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 optimization of the fermentation parameters during *B. bassiana* Bgl production as well as validates the existence of optimum points. Through this statistical optimization approach, a 5.39-fold increase was recorded in Bgl production relative to the unoptimized level of 132.71 U/mL. These results are remarkable as the optimized 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.

3.4 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 time, while cellobiose intensity decreased with reaction time (Fig. 5a). The glucose released by the Bgl over time 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 observed for the standard sugar used. Expectedly, the amount of glucose released from cellobiose increased with the incubation time and close to 100% of glucose was obtained after 24 h of incubation (Fig. 5b). The complete hydrolysis was observed after 24 h in this study, however, in previous studies by Verma et al. [44] and Pei et al. [33], *(A) niger* and *Thermoanaerobacterium thermosaccharolyticum* Bgls yielded 77% and 100% glucose within 12 h and 6 h, 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.
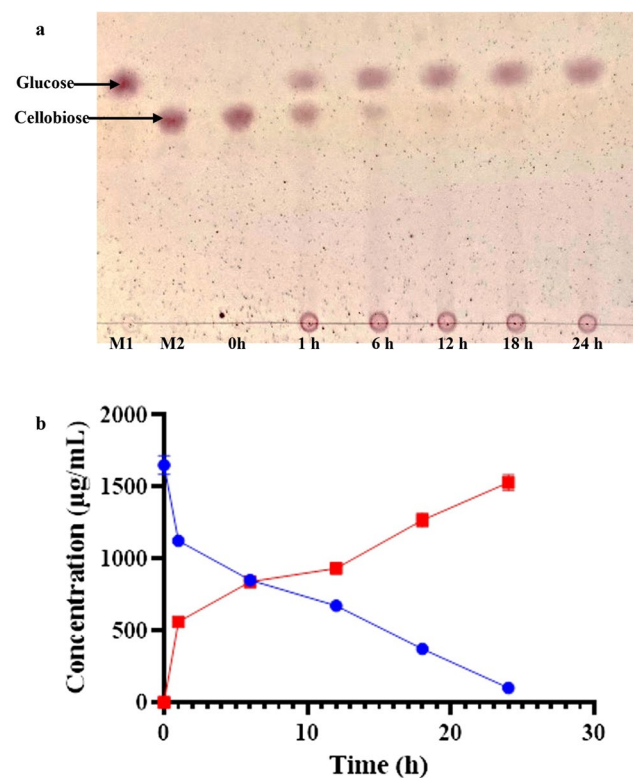
Fig. 5 a Thin-layer chromatography analysis of cellobiose hydrolysis by *B. bassiana* SAN01 β-glucosidase, M1: glucose standard, M2: cellobiose standard, lanes 3, 4, 5, 6, 7, and 8 show cellobiose hydrolysis after 0, 1, 6, 12, 18, and 24 h. **b** Cellobiose hydrolysis (blue line) and glucose production (red line) by *B. bassiana* SAN01 β-glucosidase as determined by HPLC

4 Conclusion

This study successfully optimized the production of Bgl by the novel endophytic fungus *B. bassiana* SAN01 via statistical modeling under submerged fermentation conditions. Initially, Bambara haulm was recorded as the biomass supporting the highest Bgl production; hence, it served as the major carbon source in the PDB and CCD experiments. At the end of the optimization process, an acceptable level of agreement was found between the experimental results and the statistical model. Bgl production improved by 5.39-fold compared to the unoptimized 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. The partially purified Bgl was successfully able to release glucose from cellobiose, thus indicating that Bgl can potentially be used in the hydrolysis of cellulose for biofuel production. 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, as well as improve its saccharification capabilities via optimization and protein engineering, all with the aim of enhancing its real-time industrial applications.

Declarations

Competing interests The authors declare no competing interests.

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