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Isolation, Characterization and Biodegradability Tests of Cellulase from Bacterium Obtained from the Soil of Agro Dumpsite

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Abstract
The study investigated the waste biodegradation potential of cellulase produced by bacteria isolated from soil at an agro dumpsite, along with their physicochemical properties. The bacterium was identified as *Bacillus subtilis* and subjected to extracellular cellulase production via shaking flask fermentation. The production conditions such as pH, temperature, and incubation time were optimized using random surface methodology (RSM). The response was predicted by second-order polynomial model. In addition, the enzyme produced was partially purified, characterized, and explored in the biodegradation of agricultural waste products using standard methods. Cellulase yield in the experiment varied from 3.00 to 10.30 U/mL and under the optimum conditions of pH 7.0, 40 °C and 18 hr incubation time, the value for cellulase yield was 10.70 U/mL. The enzyme purification resulted in a specific activity of 3.3 Units/ mg of protein and 20 % yield. The K_m and V_{max} of the partially purified enzyme were 3.33 mg/mL and 52.0 U/mL respectively. The optimum pH and temperature of *B. subtilis* cellulase were at pH 5.0 and 50°C respectively. The inhibitory study showed that Na^+ did not inhibit the activity of the enzyme while the enzyme activity was moderately inhibited by Mg^{2+}, Sn^{2+} and Hg^{2+}. Potassium ions (K+) strongly inhibited enzyme activity. The substrate specificity test showed that the enzyme was able to utilize other substrates. CMC, orange peel, pectin, pineapple peel and maize cob all showed 100% activity while rice husk and maize starch had an activity of 89 and 14 % respectively. The study concluded that response surface methodology (RSM) could be used to optimize bacteria cellulase production conditions and that the enzyme is suitable for various biotechnological processes such as waste management.
Keywords: Cellulose, cellulase, biodegradation, waste management, optimization.
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Introduction

A lot of cellulosic waste, comprising food, agricultural, and synthetic materials, is produced in Nigeria, a developing nation. In metropolitan areas, decomposition and incineration are the most popular methods of getting rid of these pollutants. The ensuing waste sites, smoke, and particle matter released into the atmosphere after combustion are all major causes of pollution in cities (Faulet et al. 2006, Kvavadze et al. 2009). Disposal via biological means, such as cellulolytic organisms or isolated cellulose, is gaining popularity, and studies have demonstrated cellulase biodegradation capacity (Lynd et al. 2002, Kuhad et al. 2011). Given the massive global demand for food from traditional sources, the use of wood cellulose and cellulosic waste materials as food sources for animals or even humans may become necessary. Food processors are already working with fermentation methods and cellulolytic enzyme treatments to improve the nutritional content of food and other refractory food sources or waste products (Karmakar et al. 2011). The most prevalent polysaccharide in

nature and the main building block of plant cell walls, cellulose represents an almost endless supply of renewable bioenergy. (Valjamae et al. 2001). It is the richest organic source of food, fuel, and chemicals. It has served as a key industrial raw material as well as a source of renewable energy in human life. However, its effectiveness is contingent on its breakdown into glucose. The ability to convert cellulose into biobased products and biofuels has piqued interest worldwide as a sustainable resource. Due to the high cost of their utilization methods, a large amount of cellulose waste from industrial, municipal, and agricultural sources is now being accumulated or used inefficiently (Kim et al. 2003). An enzyme known as cellulase is responsible for catalyzing the hydrolysis of cellulose, which results in the production of glucose by cleaving the cello-oligosaccharide. It is a multi-enzyme complex with a minimum of three essential elements: Zhang et al. (2006) identified three different types of endoglucanase: EC 3.2.1.4, also known as endo-1,4-β-glucanase; Exoglucanase (EC 3.2.1.91), also known as cellobiohydrolase or exo-1,4-β-glucanase; and Cellobiase (EC 3.2.1.21), often referred to as β-Glucosidase. These three crucial enzyme components are distinct both physically and enzymatically, and they all work in concert to convert cellulose to glucose throughout the entire process (Nakashima et al. 2002). Many agro-industrial processes, such as the manufacturing of biofuel, the delignification of paper pulp, the detergent industry, and the textile sector, have discovered useful uses for cellulase (Sreedharan, et al. 2015). Based on their physiological characteristics, gram-positive aerobic bacteria (*Cellulomonas* and *Thermobifida*), gliding aerobic bacteria (*Cytophaga* and *Sporocytophaga*), and fermentative anaerobes (*Clostridium* and *Ruminococcus*) (*Acetivibrio, Butyrivibrio*, and *Fibrobacter*) are the three groups of cellulose-degrading bacteria.

Anaerobic and aerobic bacteria have different techniques for hydrolyzing cellulose. Anaerobic bacteria are known to form multi-enzyme complexes (cellulosomes) that are bound to the cell surface, making recovery more difficult, whereas aerobic bacteria produce free enzymes that are better suited for cellulase production. As a result, the current study was done to identify aerobic cellulase-producing bacteria from an agricultural dumpsite, improve production conditions, and investigate the physicochemical features of the enzyme produced.

Materials and Methods

Materials

Soil and waste materials were collected from Oduduwa University dumpsite in Osun state. Ammonium sulphate, sodium arsenate, potassium sodium tartarate, ethylene diamine tetra acetic acid (EDTA) were obtained from Sigma Chemical Company Limited, St. Louis, USA. Carboxylmethyl cellulose (CMC), sodium hydroxide, sodium carbonate, sodium bicarbonate, sodium acetate, sodium chloride, sulphuric acid, acetic acid, and copper II sulphate were obtained from BDH Chemical Limited, Poole, England. All other chemicals were of analytical grade.

Methodology

Isolation and identification of the bacteria isolate

The method described by Apun et al. (2000) was adopted with minor modifications. Serially diluted soil samples were spread on CMC agar medium plates and were incubated at 50 °C for 24 hr. The plates were washed with 1 M NaCl after being submerged in an aqueous solution containing 0.1% Congo red for 15 minutes in order to visualize the hydrolysis zone. The diameter of the clear zone surrounding colonies on CMC agar was examined to identify the organisms' cellulose activity. Bergey's handbook (Holt et al. 1994) was used to identify the strains that produced cellulase.

Enzyme production medium.

The production medium contains (g/L) 0.5 gm glucose, 0.75 gm peptone, 0.01 gm FeSO4, 0.5 gm KH_2PO_4, and 0.5 gm $MgSO_4$. A 100 mL conical flask contained 50 millilitres (mL) of production medium. The flasks were sterilized in an autoclave at 121◦C for 15 minutes. After cooling, the flasks were inoculated with the overnight growing bacterial culture. The media were then cultured in a shaker incubator at 37◦C for 24 hours. The crude extract, which serves as an enzyme source, was obtained by centrifuging the culture media for 15 minutes at 5000 rpm following fermentation.

Enzyme assay

The Miller, 1959 method was employed to estimate the amount of cellulase. In summary, a reaction mixture containing 1.8 mL of prepared 0.5% substrate (carboxymethyl cellulose) and 0.2 mL of the enzyme solution was incubated for 30 minutes at 37 ◦C in a shaking water bath. Three

millilitres of DNS reagent were added to stop the reaction. The mixture was subjected to heat for five minutes for colour development. The samples' absorbance was measured at 540 nm against a blank that had the enzyme boiled and all of the reagents. The protein concentration was calculated using the Bradford method. BSA, or bovine serum albumin was used as standard.

Optimization of culture conditions for cellulase production

Response surface methodology (central composite design) was used to identify the ideal growth conditions for the synthesis of cellulase. Incubation time, temperature, and pH with coded levels of -1, 0, and +1 were the culture parameters that were optimized. The response value is the average of each experimental test's triplicates. Utilizing the Design Expert program (version 11. obtained from Stat-Ease USA, Shanghai Branch), the data were statistically analyzed. Regression analysis was used to evaluate the response function as a second-order polynomial (Wang et al. 2011), and Design Expert software was utilized for analysis of variance (ANOVA) to confirm the model's validity.

Enzyme Purification

Ion exchange chromatography

The crude enzyme was subjected to 70% ammonium sulphate precipitation. The precipitate obtained was dialyzed and layered on a CM-Sephadex C-25 cation exchanger (1.5 x 25 cm) column. The column was washed with 10 mM citrate buffer, pH 4.8 to eliminate unbound protein while elution was achieved with 0.1 M KC1 in 50 mM sodium citrate buffer, pH 4.8. At a rate of 60 mL/h, fractions (3 mL) were extracted from the column. The cellulose activity of the fractions was determined using the DNSA method. Following that, the active fractions were combined and dialysed against 50% glycerol in the elution buffer and stored at - 4 ^{o} C for further use.

Enzyme Characterization

Effect of temperature on the enzyme activity

The cellulase activity assay was conducted at temperatures ranging from 30 ºC to 100 ºC in order to examine the impact of temperature on the enzyme's activity and identify the ideal temperature for it. An aliquot of the enzyme that had been equilibrated at the same temperature was added to the assay mixture after it had been incubated at the appropriate temperature for 10 minutes.

Effect of pH on the enzyme activity

In order to determine how pH affected enzyme activity, assays for enzyme activity were conducted at three different pH ranges: 50 mM of citrate (pH 3-5), phosphate buffer (pH 6–8), and borate buffer (pH 9–11).

Effect of salts on the enzyme activity

The impact of salts on the activity of the enzyme was investigated using the following chloride salts (NaCl, KCl, $BaCl_2$, $MnCl_2$, $PbCl_2$ and $NiCl_2$) at concentrations of 1.0, 5.0, and 10.0 mM in the enzyme assay mixture

Waste Biodegradation Study

Several agroindustrial wastes, including rice husk, orange and pineapple peels, pectin, maize starch, glucose, and maize cob, were used as carbon sources in a standard cellulase assay mixture to ascertain the substrate specificity of the enzyme. The activity was calculated as a percentage of the enzyme's residual activity.

Statistical Analysis

The Design Expert program (version 11. obtained from Stat-Ease USA, Shanghai Branch). Differential analytical tools were also employed.

Results

A total of five bacterial strains were isolated from the soil. The isolates' capacity to synthesise cellulase was assessed. Of these five isolates, one was selected for further study based on its appreciable cellulase production (0.87 U/mL) potential. The most promising isolate appeared as flat, opaque, cream colonies on agar plates and gram-positive rods. It was identified as *Bacillus subtilis* based on its morphological and biochemical traits.

Optimization of Production Conditions

Analysis of the model

Tables 1 and 2 display, respectively, the experimental and predicted results for cellulase activity and the analysis of the model for production optimization. At $P < 0.05$, it was discovered that five quadratic models (AB, AC, A^2, B^2, and C^2) and three linear models (A, B, and C) were significant in this model.

Table 1. Experimental and predicted result for cellulase activity**.**

Run Order	Response U/mL	Actual Value U/mL	Predicted Value U/mL
1	3.00	3.00	3.56
2	10.00	10.00	10.11
3	6.60	6.60	6.50
4	7.50	7.50	6.95
5	6.90	6.90	6.80
6	5.60	5.60	5.60
7	10.20	10.20	10.22
8	8.70	8.70	7.94
9	4.00	4.00	4.01
10	4.50	4.50	5.12
11	7.00	7.00	7.75
12	6.50	6.50	7.14
13	5.00	5.00	5.10
14	10.21	10.21	10.22
15	10.30	10.30	10.22
16	7.10	7.10	6.49
17	3.60	3.60	2.96
18	9.60	9.60	10.22
19	9.60	9.60	10.22
20	10.10	10.10	10.22

Table 2. Response Surface Quadratic Model Analysis of Variance (ANOVA) for Cellulase Production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	117.37	9	13.04	27.28	< 0.0001*	Significant
A-Incubation time	21.10	1	21.10	44.15	< 0.0001*	
B-Temperature	3.48	1	3.48	7.28	0.0224*	
C-pH	0.0422	1	0.0422	0.0883	0.7725	
AB	4.35	1	4.35	9.10	0.0130*	
AC	2.10	1	2.10	4.40	0.0624	
BC	8.20	1	8.20	17.16	0.0020*	
A^2	48.17	1	48.17	100.77	< 0.0001*	
B^2	32.86	1	32.86	68.75	< 0.0001*	
C^2	10.13	1	10.13	21.19	0.0010*	
Residual	4.78	10	0.4780			
Lack of Fit	3.39	5	0.6783	2.44	0.1747	not significant

df – degrees of freedom; MS – mean square.
R^2 = 0.9845; R^2 adj = 0.9256; CV = 9.39 %. (*values statistically significant at P < 0.05).
Equation of the model: Cellulase yield (Activity) = +10.22 +1.24A -0.5049B +0.0556C -0.7375AB +0.5125AC – 01BC – $1.83A^2$ -$1.51B^2$ + $0.8383C^2$

The link between the independent and dependent variables is displayed in Figure 1a–c. Fig. 1a demonstrated how pH and temperature interacted while maintaining incubation time at the centre point (0). The relationship between incubation temperature and time at constant pH was depicted in Fig. 1b while Fig. 1c showed the relationship between pH and incubation time at constant temperature.

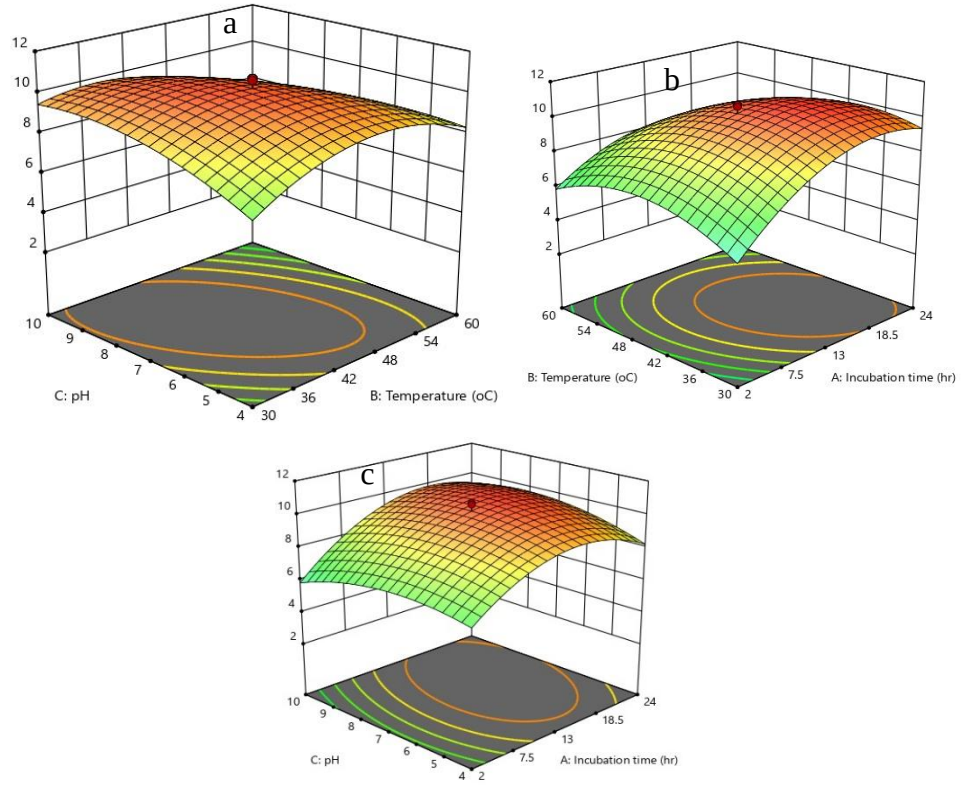

Figure 1a-c. Response surface plots showing the interaction between the tested variables -temperature, pH and incubation time on cellulase production.

Enzyme Purification and kinetic study

The purification results is summarized in Table 3 while the elution profile of the ion exchange chromatography is shown in Figure 2. The kinetic parameters of the partially purified enzyme are K_m of 3.3 mg/ml and a V_{max} of 52 U/gm

Table 3: Summary of Purification of *Bacillus subtilis* Cellulase

Fractions	**Total Protein (mg)**	**Total Activity (U/mL)**	***Specific Activity (U/mg)***	**% Yield**	**Purification Fold**
Crude	401	690	1.72	100	1
80% Ammonium Sulphate	90	252	2.8	37	1.63

Cm-Sephadex C-50	43	140.08	3.3	20	1.92

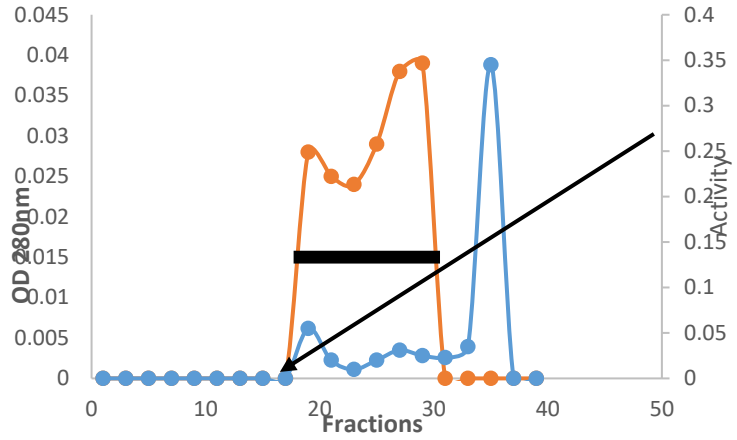

Fig 2: Elution profile of *Bacillus subtilis cellulase* on CM Sephadex c50 ion exchange chromatography. Activity Protein pooled fractions

Effect of temperature and pH on enzyme activity

The optimum temperature and pH of the enzyme was observed at 50 ^{0}C and 5.0 respectively (Figure 3 and 4)

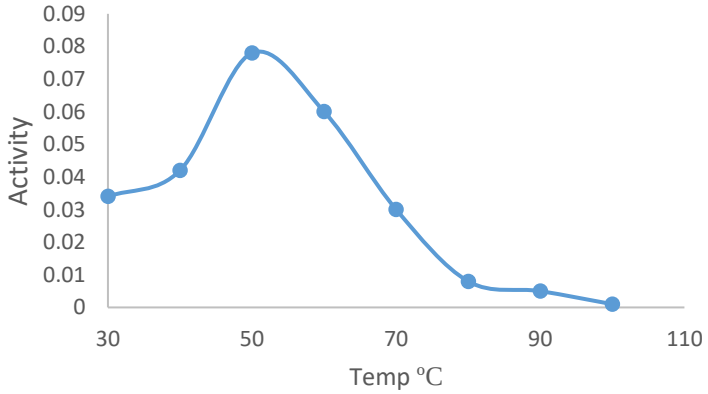

Fig 3: Effect of Temperature on *Bacillus subtilis* cellulase activity

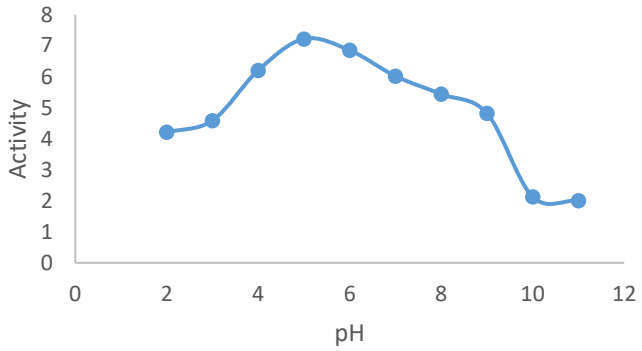

Fig 4: Effect of pH on *Bacillus subtilis* cellulase activity

Effects of metal ions on the enzyme activity

The effect of three different concentrations of Sn^{2+}, Hg^{2+}, Na^{+}, Mg^{2+} and K^{+} on the enzyme were investigated. The enzyme was markedly sensitive to inhibition by K^{+} and Sn^{2+}, but was not affected by Na^{+}, Mg^{2+} and Hg^{2+} (Figure 5)

Waste Biodegradation Study

Table 4 shows the substrate specificity of cellulase from *Bacillus subtilis.* The overall results showed that the enzyme has 100% ability to hydrolyse CMC and other cellulose based compounds but did not hydrolyse maize starch and glucose.

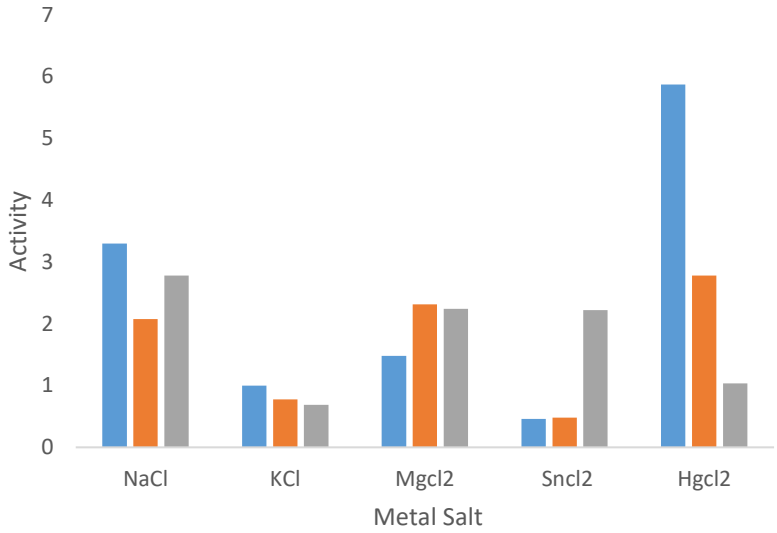

Figure 5: Effect of metal salts on *Bacillus subtilis* cellulase

Table 4. Substrate Specificity of *Bacillus subtilis* Cellulase

Substrate	**ENZYME ACTIVITY (%)**
CMC	100
Orange Peel	100
Rice Husk	89
Pectin	100
Starch Maize	14

Pineapple Peel	100
Maize Cob	100
Glucose	0

Discussion

Bacillus subtilis was isolated from the agro-waste dumpsite and was subjected to extracellular cellulase production. Studies have reported the ability of some bacteria to produce cellulase (Yin et al. 2010; Nema et al. 2015; Pachauri et al. 2017; Quadri et al.,2017; Afzal et al. 2019; Sulyman et al. 2020). The cellulase yield observed in this study is greater than the values published for cellulases from Bacillus cereus (0.104 unit/mg) (Nema et al. 2015), and lower than the cellulase from *Trichoderma longibrachiatum* (30.0 unit/mg) (Pachauri et al. 2017) *Bacillus pantothenticus* (55.28 unit/mg) (Quadri et al. 2017). Production of enzymes, especially cellulase by microorganisms is often dependent on the cultured conditions and biowaste in natural environments. In this study, cellulase production was optimized statistically. To get the most enzyme production possible from *B. subtilis*, the ideal conditions for cellulase production were identified. The cellulose yield varied (from 3.0 to 10.30 U/mL). The temperature at which *Bacillus subtilis* was incubated had an impact on its capacity to produce cellulase. The most notable influences on the reaction, as determined by the analysis of the complete data set, were temperature, incubation time, and the interaction between AB and BC, A^2, B^2 and C^2 (Table 2). Figure 1a illustrates how temperature and pH interact when incubation time is at the centre. The outcome demonstrated that when both factors increase, so does cellulose production. Nevertheless, a progressive decline in production was observed at high temperatures. Figure 1b illustrates the relationship between temperature and incubation time at constant pH. In this interaction, the yield of cellulose increases with the concentration of both factors, albeit a limited yield was seen at low concentrations. An analogous pattern was noted in the relationship between pH and incubation time (Figure 1c). Other studies have reported similar patterns to those observed in this study. (Gajdhane et al. 2016; Itakorode et al. 2022; Farzaneh et al. 2023). The accumulation of other products or metabolites, which both hinder the bacterium's growth and the production of cellulase, may be the cause of the production reduction. Alternatively, the nutrients may have run out. The optimal value of the independent variables for cellulase production was observed at pH 7, 40 ^oC and an incubation time of 18 hr, at which cellulose yield was 10.70 U/mL.

B. subtilis was purified with a specific activity of 3.3 Units/ mg of protein and a yield of 20%. Similar findings for the cellulase of different bacteria have been reported by other researchers. Darabzadeh *et al.* (2019) obtained a yield of 35.7 % from the cellulase of a mutant of *Trichoderma reesei, and a purification fold of 9.7.* Sulyman *et al.* (2020) reported a fold of 68.12 and a yield and specific activity of 3.87% and 484.3 U/mg respectively for *Aspergillus niger* cultured on *Arachis hypogaea*. The specific activity obtained in this study is higher than the value obtained from cellulase from termite *Amitermes eveuncifer* workers (Ezima *et al.* 2014) but lower than the value reported by Fagbohunka *et al.* 1997 for cellulase from heamolymph of African giant snail.

The kinetic parameters (Km and Vmax) obtained in this study were 3.33 mg/mL and 52.0 U/mL respectively. This is similar to the one reported by

Gaur and Tiwari (2015) on cellulase from *Bacillus sp* cellulase with K_m and V_{max} values of 1.923 mg/mL and 769.230 U/mL, respectively. While Sulyman *et al.* (2020) obtained 0.23 mg/mL and 9.26 U/mL for K_m and V_{max} respectively from cellulase produced by *Aspergillus niger*. *An optimum activity was observed at 50* °C for *B. subtilis* cellulase. This result is consistent with those obtained from other sources. (Thongekkaew et al. 2008; *Yin et al. 2010,* Ekwealor *et al.* 2017, Fagbohunka *et al.* 2021, Sulyman *et al.* 2020). However, a slightly higher optimum temperature (55 - 60 °C) was observed for the cellulase activity reported by Nascimento *et al.* (2009) and Jaradat *et al.* (2008). Similarly, Darabzadeh *et al.* (2019) reported a lower optimum temperature of 40 °C for cellulase of a mutant of *Trichoderma reesei.* Elsababty et al. (2022) reported a very low optimum temperature for cellulase from *B. licheniformis* strain Z9. The effect of pH on the cellulase activity from *Bacillus subtilis showed that the enzyme was very active at pH 5.0. Different pH values have been reported for the enzyme from different sources. Elsababty et al. (2022) reported a pH optimum of 7.0 for cellulase from B. licheniformis* strain Z9. Islam and Roy (2018) also reported an optimum activity at a pH of 7.0 for cellulase from *Paenibacillus* sp. Sulyman *et al.* (2020) reported a lower pH of 4 for cellulase produced by *Aspergillus niger*. Fagbohunka *et al.* (2021) working with *A. eveuncifer,* (Silverstri) soldier cellulase reported an optimum activity at pH 5. Similarly, an optimum pH of 5.0 was reported for *Thermomonospora* cellulase (George *et al.* 2001).

Metal effects showed that Na+ did not have any negative effects on the activity of enzymes. Mg^{2+}, Sn^{2+}, and Hg^{2+} all had a minor effect on cellulase activity, whereas K^+ had a significant impact. Other reports revealed varying impacts from different metals. In *B. licheniformis* strain Z9, Elsababty et al. (2022) examined the effects of metal ions and chemical reagents at a final concentration of 1 mM on enzyme activity and discovered that Mg^{2+} and Na^+ greatly inhibited the enzyme activity, whereas $Ca^{2+}$and Fe^{3+}significantly stimulated enzyme activity. Azadian et al. (2017) found that Mg^{2+} increased cellulase activity by 110 percent. The same divalent cations, however, inhibited the cellulase enzymes from *B. amyoliquefaciens* DL-3 and B. licheniformis strain MK7, according to Lee et al. (2008). *Bacillus vallismortis* RG-07 cellulase activity was increased by 10 mM Ca^{2+}, Mg^{2+}, and Na^+, but inhibited by other metal ions tested (Gaur and Tiwari 2015). According to Assareh et al. (2012), metal ions in the form of salts like $CaCl_2 \cdot 6H_2O$ shield enzymes against thermal denaturation and help stabilize their native forms at high temperatures.

The ability of cellulase from *Bacillus subtilis to hydrolyze d*ifferent substrates was determined. CMC, orange peel, pectin, pineapple peel and maize cob all showed 100% activity with the enzyme, while rice husk had an activity of 89 %. Starch maize and glucose showed activity of 14 and 0 % respectively. Other researchers have reported similar results with the enzyme having the absolute ability to hydrolyze CMC and most agro-waste materials. Sulyman *et al.* (2020) reported a 100 % relative activity for cellulase from *Aspergilus niger.* This result is similar to what was obtained in this study. Ekwealor *et al.* (2017) reported that the cellulase from *Bacillus sphaericus* CE-3 was able to utilize natural wastes like sawdust as substrate. In addition, Islam and Roy (2018) discovered that the cellulase from the strain of Paenibacillus sp. that exhibited the maximum activity against CMC exhibited low activity towards xylan.

Conclusion

B. subtilis cellulase is unique in its isolation source, which is an agricultural waste dumpsite. It demonstrated its aptitude for biomass conversion by agricultural waste hydrolysis, with peak activity at 50°C and pH 5.0. The preservation of cellulase activity in the presence of various metal ions indicated the organic ionic tolerance of B. subtilis cellulase. The optimization model incorporated increased B. subtilis cellulose synthesis. Further research is needed to improve B. subtilis cellulase production through mutagenesis and other genetic engineering techniques.

Declarations

Conflict of interest: the authors declare no competing interests.

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