J. Mod. Res. 1 (2024) 21-27

Production and activity optimization of cellulase and xylanase from the compost isolated *Paracoccus kondratievae* strain GB in Egypt

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Article information
Received:17 June 2023
Revised: 6 August 2023
Accepted: 13 August 2023

Key words
Cellulase,
Xylanase,
Lignocellulose,
Paracoccus kondratievae strain *GB*.

Abstract

The production and activity of both cellulase and xylanase from *Paracoccus kondratievae* strain GB with accession number (MW532124) isolated from compost were determined. The effect of various parameters on enzymes production including pH, temperature, carbon source, nitrogen source, incubation time, and agitation speed was investigated. Enzyme activity was also assessed by estimating the impact of temperature, pH, and incubation period. The highest value of cellulase production was accomplished at an incubation temperature of 35 °C (25.9 pKat ml^{-1}), pH 8.0 (46.96 pKat ml^{-1}), agitation speed of 150 rpm (47.8 pKat ml^{-1}), incubation period of 5-7 days (41.8 pKat ml^{-1}), CMC as a carbon source (205.8 pKat ml^{-1}) and ammonium sulphate as a nitrogen source (200.15 pKat ml^{-1}). While, xylanase was produced maximally at 35 °C (4.9 nKat ml^{-1}), pH 8.0 (6.45 nKat ml^{-1}), agitation speed of 150 rpm (4.15 nKat ml^{-1}), incubation period of 4 days (4.15 nKat ml^{-1}), xylan as a carbon source (4.5 nKat ml^{-1}) and ammonium nitrate as a nitrogen source (6.66 nKat ml^{-1}). On the other hand, the highest xylanase activity was obtained at 45 °C and pH 6.0 after 50 minutes of incubation, whereas, the highest cellulase activity was obtained at 45-55 °C and pH 6.0 after 60-70 minutes. These results show the thermophilic nature of these two examined enzymes. Our findings suggest that the thermophilic glycosyl hydrolases produced *Paracoccus kondratievae* strain GB (MW532124) might be good candidates for the degradation of lignocellulosic waste for industrial applications.

1. Introduction

The search for alternative energy sources to fossil fuels is driven by the need for renewable, sustainable, price stability, and environmental solutions. Various alternatives have emerged, but all have limitations. (1). Lignocellulosic biomass is a renewable, affordable, and carbon-neutral feedstock for second-generation biofuels. However, due to its heterogeneous nature and lignin presence, it is recalcitrant to enzyme hydrolysis. (2). Lignocellulose, a renewable organic matter source, is essential for biotechnological applications and serves as ideal substrates in woody and non-woody plants. (3). Lignocellulosic waste primarily originates from horticulture, paper-pulp, lumber, and related industries, with burning being a common method., even though its adverse effects on the environment (4).

Cellulose, the most abundant renewable bio-resource on Earth, is the primary component of plant cell walls. It is a linear homopolymer of glucose linked by β-1,4 glycosidic linkages, consisting of 7000-15000 units of D-glucose. Its regular structure allows for crystalline regions through hydrogen bonds within and between molecules. (5). It is the most abundant natural and desirable raw material that may be depolymerized and converted into a variety of products that involved in animal feed, biofuel, food, detergent, manure, paper, textiles, and waste management (6).

Hemicellulose, which makes up 15–30% of the plant cell wall, is the second polysaccharide in lignocellulose. One of the main purposes of hemicelluloses, which are embedded in plant cell walls, is to bind cellulose microfibrils to strengthen the cell wall (7). Unlike cellulose, Hemicellulose, a random amorphous structure with heteropolymers like glucomannan, glucuronoxylan, xyloglucan, xylan, and arabinoxylan(8), is significant for its potential use in bio-based products like barrier films and food additives. (9). Hemicelluloses occur mainly in plant primary cell walls and have branched and varied chemical structure. The branches are polymeric sugars. Hemicelluloses are comprised of a combination of 5- and 6-carbon ring sugars (10).

Only cellulose and hemicelluloses, which are long-chain polysaccharides that can be hydrolyzed into a mixture of fermentable pentoses and hexoses that can then be further converted to ethanol molecules, can be used for manufacturing bioethanol among the structural polymers of lignocellulose. (11).

Egypt produces 12.33 million tons of agricultural crop waste annually, with rice straw being 63.75%. Rice straw could be a renewable fuel source, replacing fossil fuels, reducing CO_2 emissions, and eliminating pollution from open-air straw burning. (12). Rice straw is a lignocellulosic material with 38% cellulose, 25% hemicellulose, and 12% lignin. Rice straw contains less cellulose and lignin and more hemicellulose than other plant biomasses, such as hardwood. (13).

Carbohydrate-Active enzymes (CAZymes) collaborate to degrade lignocellulose through oxidative, hydrolytic, and non-hydrolytic activities, typically in a coordinated cocktail.. (14). Cellulolytic activity is a result of a multi-complex enzyme system that consists of three major components: endo-glucanase

(EC 3.2.1.4), exo-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (15). Three enzymes, endoglucanase, exoglucanase, and β-glucosidase, work synergistically to efficiently hydrolyze cellulose. Cellooligosaccharides, carboxymethyl cellulose, and amorphous cellulose contain oligosaccharides that endoglucanase effectively hydrolyzes. Exoglucanase produces cellobiose or glucose, while β-glucosidase affects cellobiose and cellodextrin's non-reducing extremities. (15).

Due to the shared β-1,4-glycosidic bonds in the backbone of the hemicellulose component of plant biomass, hemicellulases frequently share comparable activities with cellulases (16). The second most prevalent natural carbohydrate, xylan, is hydrolyzed by the enzyme xylanase (endo-1, 4-β-xylanase). In order to bio convert lignocellulosic materials containing xylan into monosugars and xylooligosaccharides, (XOS) xylanases are used (17).

Numerous bacteria, actinomycetes, filamentous fungi, and yeasts have been extensively studied for their ability to degrade lignocellulosic biomass(18). Some of the most important lignocelluose-degrading microbes include *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia, Bacillus, Cellulomonas and Cellulosimicrobium* (19).

The genus *Paracoccus* belongs to the α-subdivision of non-sulfur purple bacteria, and encompasses over 34 defined species (20). *Paracoccus* species thrive in various habitats like sludge, seawater, and soil, thanks to their adaptable metabolism and ability to adapt to environmental conditions. (21). *Paracoccus kondratievae* strain GB (accession number: MW532124) is a lignocellulosic-degrading bacterial strain isolated from compost and identified based on its 16S rRNA sequence as detailed in our previous study (22). This strain exhibited potent cellulase and xylanase activities and we aim in the current study to optimize the production and activity of the cellulase and xylanase enzymes produced by *Paracoccus kondratievae* strain GB at different conditions.

2. Materials and Methods

2.1. Tested organism

Paracoccus kondratievae strain GB was obtained from the strain culture collection of MRBM (Microbial Repository of Botany and Microbiology) at the Botany and Microbiology Department, Faculty of Science, Minia University, El-Minia, Egypt.

2.2. Production of cellulase and xylanase enzymes:

Paracoccus kondratievae strain GB was tested for its ability to depolymerize cellulose and xylan in rice straw. Rice straw, is one of the most commonly present agricultural waste products in Egypt, and was used in this experiment (23). *Paracoccus kondratievae* strain GB was grown on culture medium containing (g l^{-1}): peptone, 1.0; $MgSO_4.7H_2O$, 0.3; K_2HPO_4, 2.0; $(NH_4)_2SO_4$, 2.5 and rice straw, 10.0. The culture was incubated at 37 °C with shaking at 150 rpm for 24 hrs. After incubation period, the culture was centrifuged at 5000 rpm for 20 minutes at 4 °C, and supernatant was collected for measurement of cellulase and xylanase production (24).

2.3. Enzyme assay:

Cellulase and xylanase activities in the obtained culture supernatant were measured according to (Zhang et al. 25). The reaction mixture (total volume 1 ml) contained: sodium phosphate buffer (50 mM), carboxymethylcellulose (CMC) or xylan (1%) as a substrate and culture supernatant (100 µl). The reaction mixture was incubated at 37 °C for 1 hr.

2.4. General Procedure for Determination of Reducing Sugars:

The released reducing sugars from the degradation of CMC or xylan were estimated by the 4-hydroxybenzoic acid hydrazide (PAHBAH) reagent (purchased from Sigma Aldrich), which developed by (Lever 26). The principal of this method is based on the reaction between reducing sugars and benzoic acid hydrazide in dilute alkali to form the bisbenzoylhydrazones of glyoxal and methylglyoxal. Carbohydrates that are capable of forming osazones generally react with aroylhydrazines in dilute aqueous alkali, giving colored anionic forms of the hydrazones, which measured spectrophotometrically at 410 nm (UNICO S-1200).

300 µl of sample was added to the 0.1% PAHBAH reagent (in 0.4 M NaOH, 100 mM sodium citrate). The reaction was boiled for 10 minutes and the absorbance was measured spectrophotometrically at 410 nm against a carbohydrate deficient blank. Reducing sugar concentration was estimated using a standard curve plotted for glucose and xylose of different concentrations against absorbance at 410 nm.

2.5. Optimization of culture medium for cellulase and xylanase production:

To determine the optimum conditions for cellulase and xylanase production, cultures of *Paracoccus kondratievae* strain GB were grown at different growth conditions. Unless otherwise stated**,** bacterial culture was grown on minimal salt culture medium supplemented with rice straw (1%) as the sole carbon source (pH 8) and incubated at 37^{o}C for 1 day. To determine the optimum temperature, cultures were incubated at a range of different temperatures (25-50 ^{o}C). To determine the optimum pH, the initial pH, medium was adjusted to different hydrogen concentrations from 2 to 10 pH. To determine the optimum incubation period, cultures were incubated at 37 ^{o}C and samples were collected at different incubation times. To determine the optimum culture agitation speed for enzyme production, cultures were incubated at different agitation speeds (0 - 250 rpm). To determine the preferable carbon source for enzyme production, the mineral salt culture medium was supplemented with 1% of different carbon sources (glucose, sucrose, mannose, arabinose, lactose, CMC, xylan, glucose + CMC, glucose + xylan and filter paper). To determine the preferable nitrogen source, the mineral salt culture medium was supplemented with equimolar amounts of different nitrogen sources (ammonium chloride, ammonium oxalate, ammonium sulphate, ammonium nitrate and sodium nitrate). Enzyme production was measured by quantifying the released reducing sugars at the different conditions, as previously mentioned.

2.6. Optimization of cellulase and xylanase activities:

To determine the optimum conditions for the activities of the cellulase and xylanase produced by *Paracoccus kondratievae* strain GB, culture supernatants were obtained from cultures grown on mineral salt culture medium supplemented with rice straw (1%) as the sole carbon source at the optimum conditions for the enzymes production. The culture supernatant was collected by centrifugation at (4^{o}C, 4000 rpm, 10 minutes) and used to determine the enzyme activity at different conditions.

Unless otherwise stated, cellulase and xylanase activities were determined by incubating the crude culture supernatant with 1% CMC or Breach wood xylan (respectively) in 50 mM sodium phosphate buffer (pH 7) for 1 hour. To determine the optimum temperature, the reaction was incubated at different temperatures (30-65 ^{o}C). The optimum pH for enzyme activities was determined by incubating the crude enzyme with substrate in the presence of buffers of different pH values (2-10) (Hydrochloric Acid-Potassium Chloride Buffer, acetate buffer , Phosphate Buffer and Sodium Carbonate / Sodium Bicarbonate Buffer).

To determine the optimum incubation period for enzyme activity, the reaction mixture was incubated for different incubation times (10-80 minutes). Enzyme activity was determined by measuring the amount of released reducing sugars (27).

2.7. Data analysis:

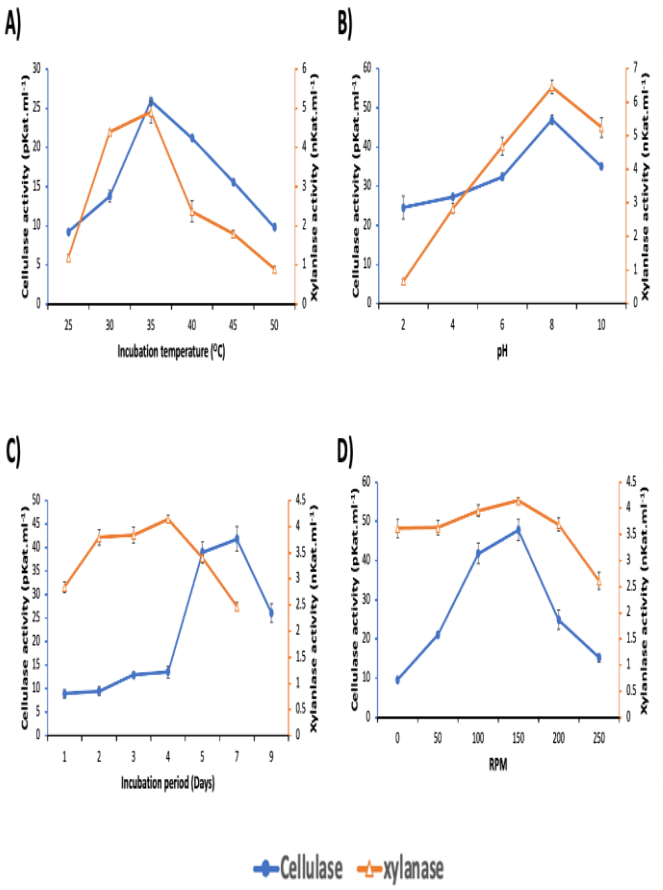
The experimental data were expressed as means ± standard error (SE) of three independent enzymatic reactions. Enzyme activity was measured by Katal. One Katal refers to an enzyme catalyzing the reaction of one mole of substrate per second. Picokatal (pKat) and nanokatal (nKat) were used to measure cellulase and xylanase activities, respectively.

3. Results

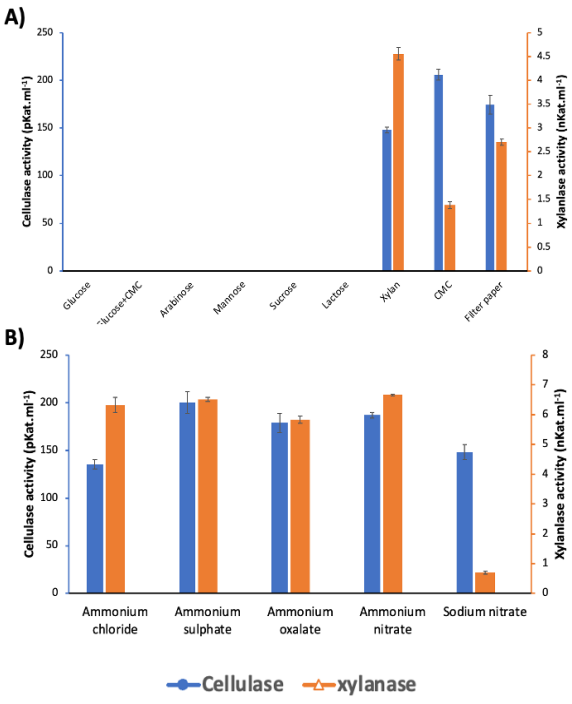
3.1. The optimum conditions for cellulase and xylanase production:

Paracoccus kondratievae strain GB (accession number: MW532124) was grown under different conditions, and the cellulolytic and xylanolytic activities were quantified to determine the optimal conditions for enzyme production. It was observed that the optimum incubation temperature for the production of both cellulase (25.9 pKat ml^{-1}) and xylanase (4.9 nKat ml^{-1}) was 35 °C **(Fig. 1A)**. The highest production for both cellulase (46.96 pKat ml^{-1}) and xylanase (6.45 nKat ml^{-1})was obtained at pH 8.0 **(Fig. 1B)**. The highest cellulase production (39.0-41.8 pKat ml^{-1}) was obtained after 5-7 days of incubation, whereas the xylanase production gradually increased after the 2nd day (3.8 nKat ml^{-1}) to reach the maximum production on the 4th day (4.15 nKat ml^{-1}), as shown in **Fig. 1C**. The optimum agitation speed for the production of both enzymes was 150 rpm **(Fig. 1D)**. Both cellulase and xylanase were only produced when polysaccharides (xylan, CMC and filter paper) were used as the sole carbon source **(Fig. 2A)**. No enzyme production was detected when soluble sugars (glucose, arabinose, mannose, sucrose and lactose) were used as carbon sources. The highest cellulase production (205.8 pKat ml^{-1}) was obtained when CMC was used as the sole carbon source, whereas the highest xylanase production (4.5 nKat ml^{-1}) was obtained on xylan as a carbon source **(Fig. 2A)**. The highest cellulase production was obtained when ammonium sulphate was used as a nitrogen source (200.15 pKat ml^{-1}), as shown in **Fig. 2B**. The highest xylanase production was obtained when ammonium chloride, ammonium sulphate or ammonium nitrate were used as a nitrogen source (6.32 - 6.66 nKat.ml^{-1}) **(Fig. 2B)**. When ammonium oxalate was use as a nitrogen source, xylanase production (5.84 nKat.ml^{-1}) was slightly reduced. However, sodium nitrate drastically reduced the xylanase production (0.69 nKat ml^{-1}).

3.2. The optimum conditions for cellulase and xylanase activities:



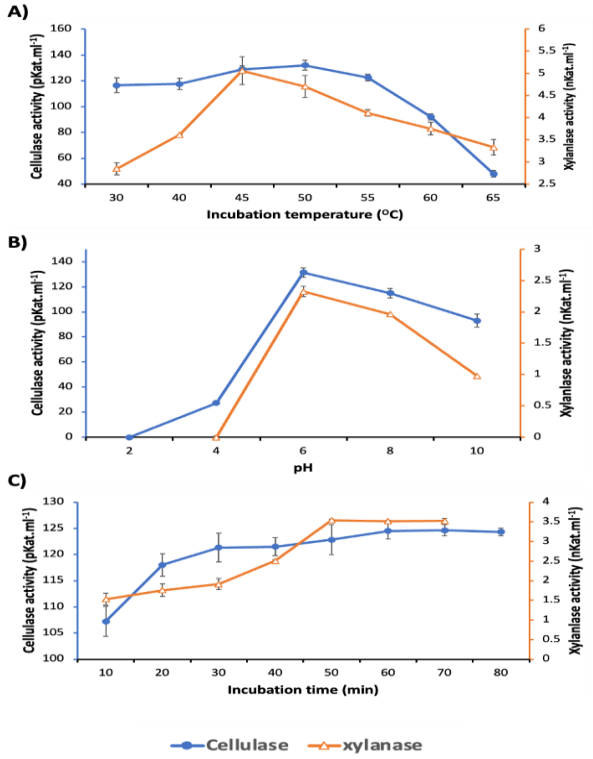
Figure(1): Effect of growth conditions on cellulase and xylanase production by *Paracoccus kondratievae* strain GB. (Incubation temperature "A", Initial pH of culture medium "B", incubation time "C" and agitation speed "D"). The bars represent the means ± standard error of three independent enzymatic reactions.



Figure(2): Effect of carbon and nitrogen sources on cellulase and xylanase production by *Paracoccus kondratievae* strain GB. (Carbon source "A" and nitrogen source "B"). The bars represent

the means ± standard error of three independent enzymatic reactions.

At 50 °C the best activity value of cellulase was 131.95 pKat ml^{-1}, which represent the optimum temperature for enzyme activity **(Fig. 3A)**, on the other hand, the optimum temperature for xylanase activity was 45°C (5.06 nkat.ml^{-1}) **(Fig. 3A)**. The highest activity of both cellulase (131.44 pKat ml^{-1}) and xylanase (2.32 nKat ml^{-1}) was observed at pH 6.0 **(Fig. 3B)**. The highest xylanase activity was attained quickly after 50 minutes of incubation period (3.54 nKat.ml^{-1}), whereas, the highest cellulase activity (124.6 pKat.ml^{-1}) was obtained after 60 and 70 minutes **(Fig. 3C)**.



Figure(3): Optimization of cellulase and xylanase activities. Optimum temperature "A", optimum pH "B" and optimum incubation time "C". The bars represent the means ± standard error of three independent enzymatic reactions.

4. Discussion

Lignocellulosic biomass is an alternative and cost effective resource for the production of biofuels due to its abundance and renewability (28). The leaves, stems, and stalks from materials like maize fiber, corn stover, sugarcane bagasse, rice hulls, woody crops, and forest residues are just a few examples of the sources for lignocellulosic biomass.

Cellulose is the main structural constituent of plant cell wall, while hemicellulose is the second most abundant component of lignocellulosic biomass (8). Cellulases are considered as one of the key components in the technologies for successfully converting cellulosic biomass to fermentable sugars. Cellulases are used in a variety of industries including: brewing, wine, pulp and paper, textiles, detergents, and feed.

The bioconversion of lignocellulosic material and agricultural waste into fermentative products, the clarification of juices, an increase in the consistency of beer, and the digestibility of animal feed stock are numerous potential applications for xylanases (29). Exploiting xylanase's efficacy in biotechnology is more critical than previously, because it is used to saccharify xylan in agricultural waste and processed foods. While microbial cellulases have demonstrated their potential use in a number of industrial fields, including textile, pulp and paper, laundry, brewing, agricultural, and biofuel (15).

Several bacteria have been reported to produce both cellulases and xylanases (30). Bacteria are more predictable because they can adapt to various environmental conditions, grow quite rapidly compared to other microbes, and produce a complement of highly stable enzymes that are exceptionally effective sources of individually valuable enzymes (31).

We previously isolated *Paracoccus kondratievae* strain GB (accession number: MW532124) (22). In this study, we aimed to determine the optimum conditions for the production and activity of both cellulase and xylanase from *Paracoccus kondratievae* strain GB.

The assessment of the medium's composition is a crucial element in the cultivation of microorganisms. The cultivation medium should encompass all critical nutrients required by microorganism (32). The biosynthesis of enzymes has been subject to influences from diverse physical and chemical factors, including the constituents of the culture medium, duration of the incubation, pH level, temperature, and quality of carbon and nitrogen sources (33).

Multiple microbial cellulolytic enzymes are needed for the complex process of lignocellulosic material degradation (34). The depolymerization of the polysaccharide components into sugar monomers is an intensive procedure that incorporates a variety of enzymes (35).

The inherited characteristics of the organism, the physiochemical parameters, the components of the fermentation medium, and their quantities all affect the microbial synthesis of enzymes. Therefore, it is critical to optimize the culture conditions in order to maximize the yield of ligocellulolytic enzymes. Several authors claimed that improving parameters of bioprocess led to an increase in enzyme production (36)

According to different previous reports, both temperature and pH have an impact on the quantity of the produced cellulases and xylanases (37). In the current investigation, *Paracoccus kondratievae* strain GB produced the highest levels of two tested enzymes at 35 °C. It was discovered that the temperature affected the production of extracellular enzymes, possibly by altering the physical characteristics of the cell membrane. According to the findings of hydrogen concentration, the pH of the culture medium affected the synthesis of enzymes. Hence, the maximal production for cellulase (46.96 pKat.ml^{-1}) and xylanase (6.45 nKat.ml^{-1}) was obtained at pH 8. These results are consistent with (Kelly et al. 38) , where the bacterium *Paracoccus kondratievae* strain GB is alkaliphilic and thermotolerant, growing at the range of 30-50 °C and pH of 7.5-10.5 showing optimum peaks at 38-42 °C and pH 8.0-9.0.

According to several studies, different microorganisms have varied optimum temperature and pH level for producing cellulase and xylanase. (Sinjaroonsak et al. 39) recorded maximum cellulase and xylanase activities at 40 °C for *Streptomyces* at pH 6.5, whereas, (Sohail et al. 40) and (Farinas et al. 41) reported optimum temperature of 35 °C for cellulase and xylanase production at pH 4.0 by *Aspergillus niger* in submerged fermentation. On the other hand, different strains of *Bacillus* sp. gave a maximum yield of cellulase production at incubation temperatures of 50 °C and at pH 6.0-8.0 (42).

In order to observe the rate of cellulase and xylanase synthesis by *Paracoccus kondratievae* strain GB under ideal growing conditions, time course experiments were conducted. The data of this study show that the production of cellulase rose gradually until 5-7 days, whereas, the activity for xylanase production was observed to be at its peak on day 4 of incubation. Our observations corroborate the report of (Asem et al. 43), who noticed that the optimum incubation period for cellulase was also within 5 days in the early stationary phase of growth and maximal xylanase activity after the 3rd day.

The impact of agitation speed on cellulase and xylanase production by *Paracoccus kondratievae* strain GB showed positively effect. It has been noted that agitation can impact the amount of aeration and nutrient mixing in the fermentation medium (44). Maximum cellulase and xylanase production was recorded at 150 rpm with higher enzyme activities (47.8 pKat ml^{-1} and 4.15 nKat ml^{-1}). Our data agree with those of (Fatokun et al. 37) who reported that the best agitation rate for xylanase was 150 rpm and for cellulase was between 100 and 150 rpm.

Nutrient supplies have been reported to be key elements in the synthesis of cellulase and xylanase, and carbon source is thought to be an essential nutrient affecting enzyme production (45). We tested the impact of different carbon sources, such as glucose, sucrose, mannose, arabinose, lactose, CMC, xylan, glucose + CMC, glucose + xylan and filter paper on enzyme production. The results indicated that CMC, xylan and filter paper induced both cellulase and xylanase production, while the soluble sugars suppressed enzyme production. This demonstrated that the enzymes appeared to be inducible and needed CMC, xylan, or lignocellulosic material for the culture to induce enzyme synthesis. (Peixoto-Nogueira et al. 46) investigated the xylanase production from *Aspergillus niveus* and *Aspergillus fumigatus* and reported high levels of xylanase production on agricultural residues, including wheat bran and corn cobs. The authors also reported that galactose and sucrose did not increase the synthesis of xylanase. When sucrose was introduced in the medium as a carbon source, (Abdelwahed et al. 47) discovered that the synthesis of cellulase free xylanase from *Streptomyces halstedii* NRRL B-1238 was suppressed.

In the production of cellulase and xylanase, nitrogen sources are just as crucial as carbon sources. We tested different nitrogen sources, such as ammonium chloride, ammonium oxalate, ammonium nitrate and sodium nitrate were replacing ammonium sulphate in culture medium with maintaining equimolar amount of nitrogen. Ammonium sulphate was the most effective nitrogen source for cellulase production (200.15 $pKat.ml^{-1}$), and this is consistent with (Iram et al. 48), While in xylanase production, ammonium nitrate was the preferable nitrogen source for enzyme production (6.66 $nKat.ml^{-1}$). According to (Otero et al. 49), one of the most effective nitrogen sources for the production of xylanase is ammonium nitrate, followed by ammonium sulphate.

For determining the most suitable temperature, pH, and reaction incubation time for enzyme activity, the cellulolytic and xylanolytic activity of the culture filtrate of *Paracoccus kondratievae* strain GB were monitored under various parameters of reaction conditions. The highest cellulolytic and xylanolytic activity were obtained when the CMC and xylan degradation reaction were performed at pH 6.0. This result conflicts with the pH that promotes the production of enzymes (pH 8.0). Although the highest enzyme activity was achieved at pH 6.0, these enzymes produced by the isolate displayed a significantly high activity at a pH range from 6.0 to 8.0. This study showed that those enzymes are pH adaptable through a wide range, therefore can be used in a variety of industrial applications. This result agrees with those reported for enzymes produced by many microorganisms (42).

The highest cellulolytic activity was obtained when the enzymatic reaction was incubated at 45-55 °C, whereas xylanase activity was optimal at 50 °C, indicating the thermophilic nature of these enzymes. However, the optimum temperature for the activity of the two enzymes were higher than the optimum temperature (35 °C) for the enzyme production by *Paracoccus kondratievae* strain GB. These findings are consistent with those reported for other microbial enzymes (41). Increasing the reaction incubation time greatly increased cellulase (50 minutes) and xylanase (60 minutes) enzyme activities, which was also previously reported by (Sharma and Chand 50).

The cellulase and xylanase produced by *Paracoccus kondratievae* strain GB are suitable candidates for industrial and biotechnological uses due to their thermophilic nature, broad pH range, and capability to degrade various agricultural waste products. However, further investigation is needed to clone and express the genes encoding the cellulases and xylanase produced by *Paracoccus kondratievae* strain GB and to study enzyme kinetics on the pure recombinant proteins. Our study group is still conducting additional studies on the purification and commercial use of cellulase and xylanase enzymes.

Conclusion

Paracoccus kondratievae strain GB isolated from compost was found to have optimal cellulase and xylanase production and activity in the current research investigation. This conclusion supports the effective use of bacterial cellulase and xylanase in applications relevant to food and textile industry. Thus, motivation for additional study into the purification and characterization of such enzymes will be processed into consideration.

Financial Disclosure Statement: “The authors received no specific funding for this work."

Conflict of Interest: The authors declare that they have no conflict of interest.

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