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Substrate specificity and catalytic properties of glucoamylase from *Leohumicola incrustata*

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Abstract
Glucoamylase from fungi has proved to be a very useful enzyme for industrial processes by improving yields of food and starch products. This study determined specific and catalytic properties of purified glucoamylase obtained from a fungus, Leohumicola incrustata (MF374380). The enzyme was produced using submerged fermentation method and crude enzyme obtained was centrifuged and purified. Avicel, beechwood (BW) xylan, carboxymethylcellulose (CMC), colloidal chitin, and soluble starch as well as glycogen, amylose, amylopectin, maize starch (raw), and blocked p-nitrophenyl-a-D-maltoheptaoside (BpNPG7) were used for substrate specificity assay. Michaelis-Menten plot was also used to determine catalytic properties of the enzyme. Findings revealed that the purified glucoamylase (37.6 U/mg protein) was specific for only starch while the enzyme could not hydrolyze Avicel, BW xylan, CMC, colloidal chitin and BPNPG7. Michaelis-Menten plots results showed a K_m value of 0.70 mg/mL on glycogen, followed by amylopectin, amylose, and raw maize starch, with K_m values of 0.76, 1.05, and 1.77 mg/mL, respectively. The K_{cat} values for raw maize starch, amylopectin, glycogen, and amylose were 73, 66, 57, and 32 s^{-1}, respectively. Therefore, the study revealed that enzyme from L. incrustata exhibited strong substrate affinity and catalytic properties. The study underpins the need to include this organism when prospecting for future industrial glucoamylase production.

Keywords: Amylase, Fungus, Catalytic property, Starch, Substrate specificity

INTRODUCTION
The unique natural traits and intrinsic selectivity of fungi present in the ecosystem and soil environment have informed the continuous exploration of these organisms for the benefits of man. Their capacity to produce enzymes, which help breakdown substrates into smaller, more obtainable forms, has been connected to the regulatory mechanism of fungal raw-starch-degrading enzyme (Ning *et al.,* 2023). One crucial step in making effective use of starchy waste

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materials prevalent in nature is the utilization of microbes to hydrolyze starch (Das and Varma, 2011; Ja'afaru, 2013). Granules of glucose polymers make up starch, which is an insoluble, non-structural carbohydrate found in plants (Pfister and Zeeman, 2016). Generally, amylases help fungi to breakdown composite starchy materials into smaller products (Tanaka *et al.,* 2020).

For many years, fungi have been extensively used in industrial applications, and their potential for biotechnological use in the bioeconomy has been encouraging. Therefore, a lot of work is being done to characterize improved enzymes from new microbial sources. These techniques are helpful in screening a large number of fungal isolates for enzyme production and often enable quick identification of either a positive or negative enzyme producer (Arif *et al.,* 2024). Biocatalytic proteins such as amylases hydrolyze starch into a variety of products, including smaller polymers made of glucose units and dextrin (Natasa *et al.,* 2011; Mohammed and Mastan, 2013).

Microbial enzymes are useful biotechnologically tools because of their affordability, high yields, ease of optimization, stability, and increased catalytic activity (Gurung *et al.,* 2013; Fasim *et al.,* 2021; Sharma *et al.,* 2022). Microorganisms are a source of microbial amylases, which are now widely available for commercial purposes and have almost taken over the role of chemical hydrolysis in the starch manufacturing industries (Hussain *et al.,* 2013).

Amylases are usually classified into three viz; gamma amylase, beta amylase, and alpha amylase. Alpha amylase converts starch's α, 1-4-glucosidic connections into an endo form and generates oligosaccharides (Parka and Son, 2011). Beta amylase acts on the non-reducing end to cleave maltose, or two glucose units, at a time (Bijttebier *et al.,* 2007) while gamma amylase breaks down the last α-(1-4) and α-(1-6) glycosidic bonds at the non-reducing end of amylose and amylopectin, releasing glucose (Adeoyo *et al.,* 2019).

There is increased demand for amylases that readily convert starchy substrates industrial products such as fruit juice, beverages and animal feeds. Considering the importance of readily available substrates and efficient production of amylases for industrial processes and the fact that the potential of amylases in starch industry needs to be extensively explored, the present study was carried out to use novel fungal strains for amylase production for possible application in starch-based industry. Hence the study employed the use of *L. incrustata* as a source for glucoamylase with strong substrate specific and catalytic properties.

MATERIALS AND METHODS

Culture

Culture used for this study (*Leohumicola incrustata* - MF374380) was obtained from the Department of Biochemistry and Microbiology at Mycorrhizal Research Lab., Rhodes University, Grahamstown.

Enzyme screening

A Modified Melin Norkrans (MMN) agar medium which had the following composition (g L^{-1}): starch (1%, w/v); malt extract 3.0; $(NH_4)_2HPO_4$ 0.25; $MgSO_4.7H_2O$ 0.15; $CaCl_2$ 0.05; NaCl 0.025; $ZnSO_4.7H_2O$ 0.003; thiamine-HCl 100 µg l^{-1} and 1.2 ml of $FeCl_3$ (1%, w/v) and 1.5% agar (Adeoyo *et al.,* 2019). After 15 minutes of autoclaving at 121°C, the cooled medium (around

45°C) was mixed with 0.05 g/L of chloramphenicol before being poured into plates to stop the growth of bacteria. Each Petri plate was filled with roughly 20 mL of the medium, which was then let to set and solidify before being inoculated with a 6 mm *L. incrustata* mycelial plug. For 21 days, the plates were incubated at 28°C. Following incubation, plates were immediately flooded with a 10% v/v iodine solution to see the zone of clearance.

Enzyme production, purification, and assay

For enzyme production, a liquid medium of Modified Melin-Norkrans (MMN) was used. A rotary incubator shaker was used for incubation, after 21 days incubation period at 28°C, the culture was filtered and centrifuged at 10000 x *g* for 30 min (under a temperature of 4°C) to obtain a cell-free crude enzyme filtrate. The crude enzyme filtrate was purified using a method described by Adeoyo *et al.* (2018). Ammonium sulphate [$(NH_4)_2SO_4$] was used to concentrate the cell-free crude filtrate. After that, it was centrifuged for 15 minutes at 4°C at 10,000 x g. A 0.1 M acetate buffer (pH 5.0) was used to dialyze 10 mL of the precipitated enzyme. Enzyme activity was determined by using 1% starch in acetate buffer (pH 5.0) for one hour at 45°C. Singh *et al.* (2014) method of using 3,5-dinitrosalicylic acid was used to determine amount of reducing sugars produced under standard assay conditions. One unit of activity was defined as the quantity of glucoamylase that liberated one micromole of glucose per minute (U/mL/min).

Determination of protein content

The protein content was estimated according to a method described by Adeoyo *et al.* (2018). Protein concentration was determined using bovine serum albumin (BSA) as a standard. The protein optical density was measured at 595 nm.

Laboratory scale extraction of starch from maize

Extraction of starch from maize (*Zea mays*) was performed as described by Sheriff *et al.* (2012) with slight modification. A 60 mL of 1% sodium metabisulfite solution was used to steep 10 g of cleaned yellow maize grains for 72 hours at 45°C. The pericarp and germ were then manually removed. A vortex-type tissue homogenizer (Ultra Turrax, 20000 rpm) was used to homogenize the separated endosperms for 2 minutes after they had been placed in a 250 mL centrifuge tube with 100 mL of distilled water. The volume was made up to a total volume of 1000 mL, the homogenized slurry was vacuum-filtered through multiple washes with a muslin cloth until the wash water turned clear. Two techniques that included sedimentation and slurry centrifugation methods were used to extract starch from maize (Ji *et al.*, 2004).

$$\text{Yield of Starch} = \frac{\text{Dry Weight of Starch}}{\text{Dry Weight of Whole Maize}} \times 100$$

Substrate affinity assay

Substrate such as starch, carboxymethylcellulose (CMC), Avicel, beechwood xylan, or colloidal chitin (1%) was used to determine glucoamylase-substrate affinity. The glucoamylase also was tested on some common amylase-degrading substrates such as glycogen, amylose, amylopectin, maize starch (raw), and benzylidene blocked *p*-nitrophenyl-α-D-maltoheptaoside (BPNPG7).

Enzyme kinetics

Utilizing a Michaelis-Menten type substrate saturation curve, the rate of hydrolysis was measured to ascertain glucoamylase K_m and V_{max} values. Each substrate (raw maize starch, amylose, amylopectin, or glycogen) is present in the reaction mixture at 0 to 10 mg/mL

concentrations under pH of 5.0. Michaelis constant (K_m) was calculated. Software called KaleidaGraph was used to analyze the results.

Data analysis

One-way analysis of variance (ANOVA) was used. All experiments were carried out in triplicate and standard errors of the means (±SEM) were used for the error bars.

RESULTS AND DISCUSSION

Enzyme screening on starch agar plate

The zone of hydrolysis on starch-agar medium revealed 12±1.53 mm hydrolytic zone (Fig. 1) while specific activity value for the purified glucoamylase was 37.6 U/mg protein on soluble starch substrate (Fig. 2). This is similar to the study of Jebor *et al.* (2014) who reported terminal specific activity of glucoamylase to be 31.214 U/mg for *Aspergillus niger*.

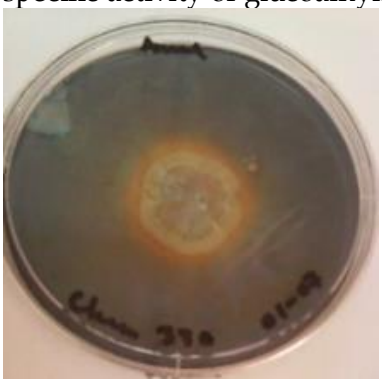

Fig. 1: Hydrolytic zone of *Leohumicola incrustata* on starch agar plate

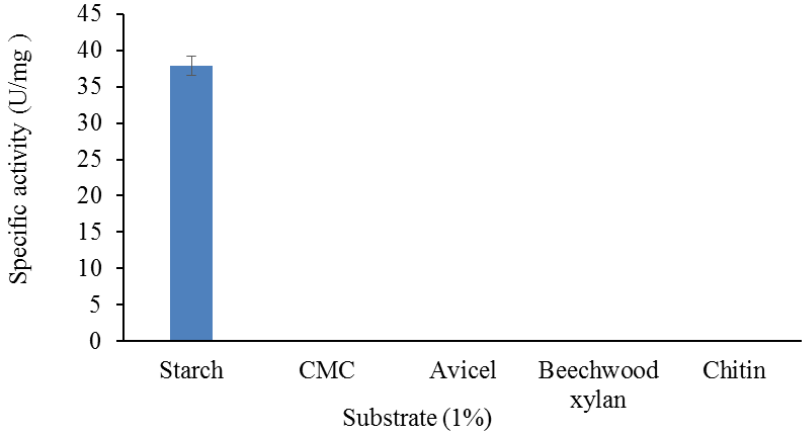

Fig. 2: Substrate specificity study and determining glucoamylase activity on different substrates to confirm for other co-produced enzymes. All error bars are represented as the standard errors of the means (±SEM).

Substrate specificity

The substrate specificity showed that starch was the only substrate that the glucoamylase could hydrolyse and no activity was recorded for the other substrates (Fig. 2). Furthermore, when some amylase-degrading substrates were employed, it was noticed that the enzyme hydrolysed all the substrates except BPNPG7 (Fig. 3) while this study also showed that only α-amylase could hydrolyse BPNPG7, not the glucoamylase (Fig. 4).

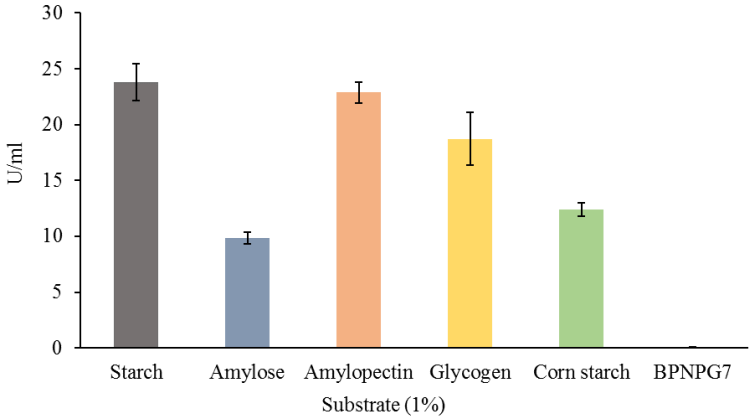

Fig. 3: Substrate specificity study and determining glucoamylase activity on the various amylase-degrading substrates to investigate glucoamylase affinity for each substrate. All error bars are represented as the standard errors of the means (±SEM).

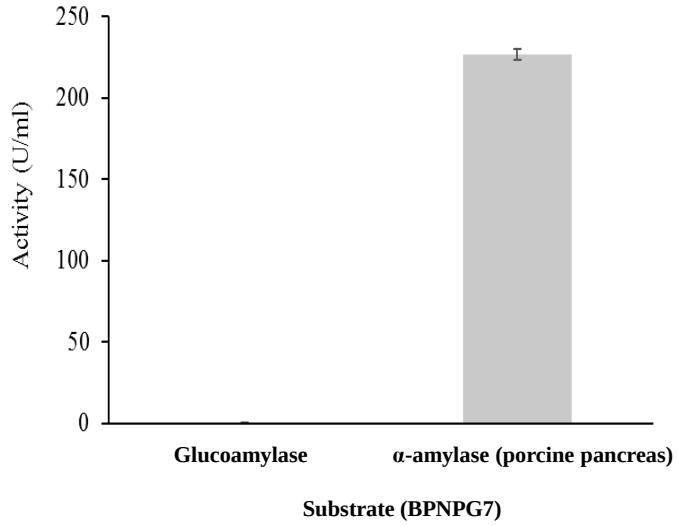

Fig. 4: Enzyme activity of glucoamylase (*Leohumicola incrustata*) and α-amylase (porcine pancreas) on BPNPG7 (blocked *p*-nitrophenyl-α-D-maltoheptaoside). All error bars are represented as the standard errors of the means (±SEM).

Starch yield (maize)

The result of starch yield in this investigation indicated that an average starch yield of 14.3% was obtained with the slurry centrifugation method, while sedimentation at 4°C for 2 h gave a 22% starch yield after 72 h of steeping and homogenisation (Table 1). The maximum 22% raw starch yield after sedimentation at 4°C was utilized as one of the substrates. This supports some findings that starch extracted using sedimentation (letting the slurry settle at 4°C for at least two hours) produced a higher yield of starch than starch extracted via centrifugation (Ji *et al.,* 2004; Sheriff *et al.,* 2012).

Table 1: Sedimentation methods and raw starch yield from corn after steeping and homogenisation

Sedimentation method	Starch yield (%)
Sedimentation at 4°C	22.0±2.06
Centrifugation at 6000 x *g*	14.3±1.63

All values are presented as means ± SEM, n = 3 per treatment

Enzyme kinetics (substrate affinities)

Michaelis-Menten plots using KaleidaGraph. K_m represents the Michaelis constant of the enzyme and V_{max}, the maximum velocity achieved by the enzyme-starch reaction (Figures 5 to 8). The K_m and V_{max} demonstrated the affinities between starch-degrading substrates and the glucoamylase. The enzyme showed a K_m value of 0.70 mg/mL on glycogen, followed by amylopectin, amylose, and raw maize starch with K_m values of 0.76, 1.05, and 1.77 mg/mL, respectively. Similarly, K_{cat} values of 73, 66, 57, and 32 s^{-1} were obtained for raw maize starch, amylopectin, glycogen, and amylose, respectively (Table 2). Glycogen, amylopectin, amylose, and raw maize starch were the substrates with varying affinity values. The values reported by Slivinski *et al.* (2011) on low substrate utilization to achieve enzyme-substrate saturation are consistent with the data obtained in this study.

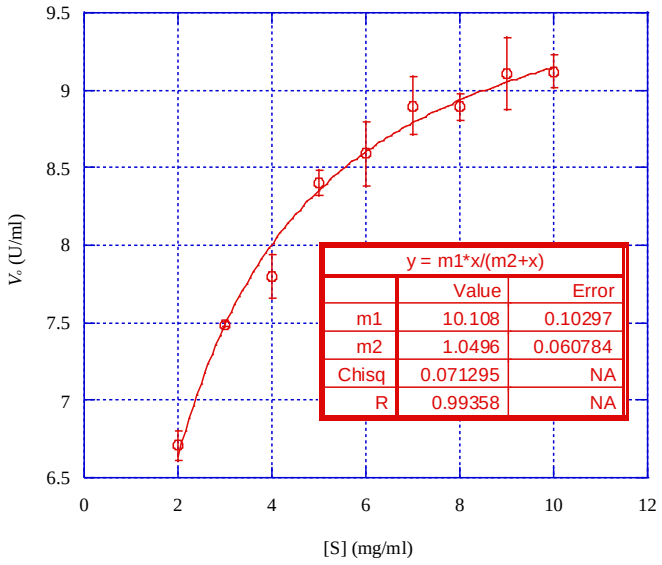

Fig. 5: Michaelis-Menten type kinetics of purified glucoamylase enzyme versus amylose concentrations. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

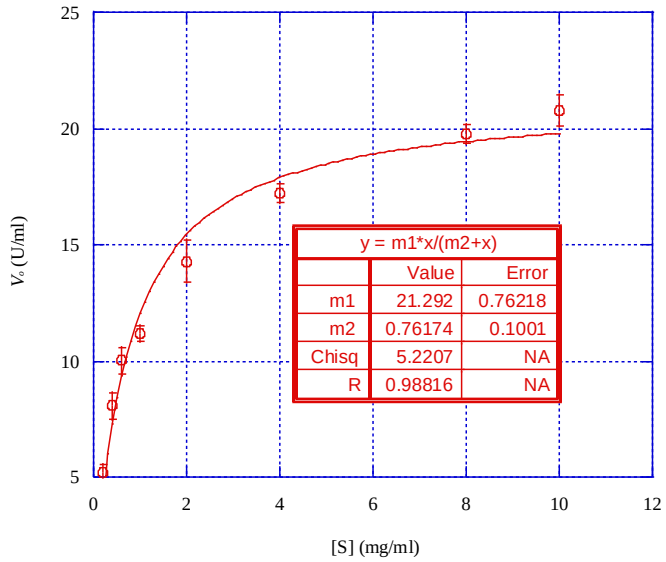

Fig. 6: Michaelis-Menten type kinetics of purified glucoamylase enzyme versus amylopectin concentrations. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

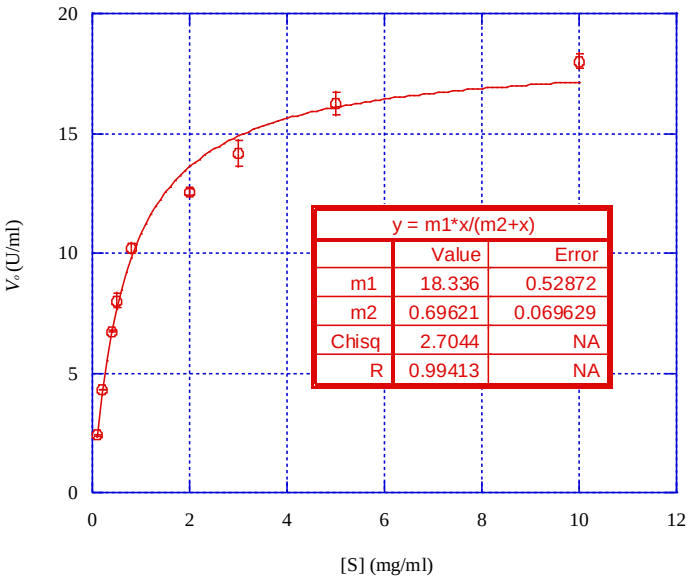

Fig. 7: Michaelis-Menten type kinetics of purified glucoamylase enzyme versus glycogen concentrations. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

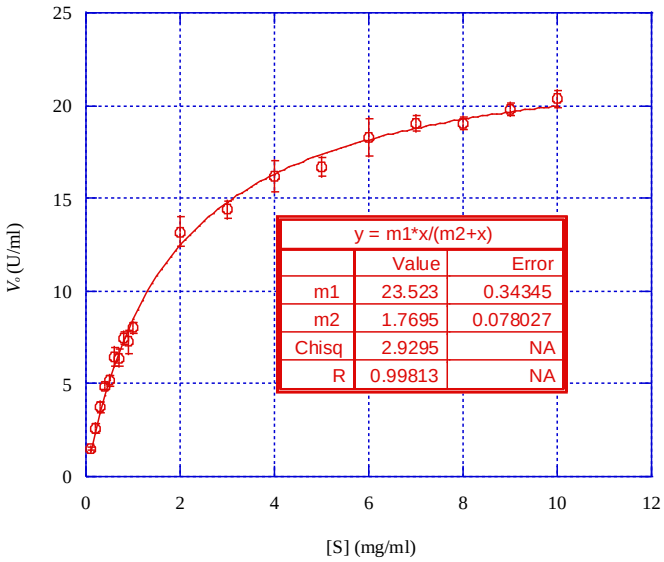

Fig. 8: Michaelis-Menten type kinetics of purified glucoamylase enzyme versus raw maize starch concentrations. All error bars are represented as the standard errors of the means (SEM) (m1 = V_{max} and m2 = K_m).

Table 2: Kinetics study of glucoamylase against various substrates analysed using Michaelis-Menten plots

Substrate	K_m (mg/mL)	V_{max} (µmol/min/mL)	Catalytic constant, K_{cat} (s^{-1}) = $\frac{V_{max}}{[E_t]}$	Specificity constant (s^{-1} mg^{-1} mL^{-1}) = $\frac{K_{cat}}{K_m}$
Amylose	1.05	10.11	32	30
Amylopectin	0.76	21.29	66	87
Glycogen	0.70	18.40	57	81
Raw maize starch	1.77	23.52	73	41

The glucoamylase enzyme was produced under liquid-state fermentation condition. There was no discernible hydrolytic activity against colloidal chitin, beechwood xylan, Avicel, or CMC. As a result, the specificity observed with glucoamylase was limited to the glycosidic linkages of α-(1,4 or 1,6) rather than β-(1,3 or 1,4). The catalytic capacity of α-amylase from pig pancreas and glucoamylase from *Leohumicola* sp. on BPNPG7 (a substrate specific for measuring α-amylase activity) showed that the glucoamylase was an exo-acting enzyme that was unable to hydrolyze BPNPG7 (McCleary *et al.*, 2002; Cornaggia *et al.*, 2016; Megazyme, 2017). The K_m and V_{max} expressed the kinetics of glucoamylase, where K_m is the substrate concentration at ½V_{max} while V_{max} was the maximum rate achieved by an enzyme at enzyme-substrate saturation point.

Moreover, α-1,4 glucosidic linkage (amylose) and α-1,6 glucosidic linkage (amylopectin) were easily hydrolyzed into glucose by the amylase, according to reports on glucoamylase from *L. shimeji* (ECM fungus) and *Rhizopus oryzae* (Hur *et al.*, 2001; Kusuda *et al.*, 2004; Abdelwahab, 2015). In contrast, because this enzyme is exo-acting in nature, the BPNPG7 was unable to be hydrolyzed. It is noteworthy to mention that glucoamylase demonstrated its potential for

hydrolyzing starch-based feedstock in the commercial setting by hydrolyzing the raw starch starch from maize (or corn) with a K_m value of 1.77 mg/mL and a K_{cat} of 73 s^{-1}. It was evident that other similar substrates can be hydrolyzed by the enzyme based on K_m values of 0.70, 0.76, 1.05, and 1.77 mg/mL obtained for glycogen, amylopectin, amylose, and raw maize starch, respectively.

The findings on substrate affinity are consistent with the Michelin *et al.* (2008) who reported that glucoamylase produced by a thermotolerant fungus (*Paecilomyces variotii*) preferentially hydrolyzed amylopectin, glycogen and starch, and to a lesser extent malto-oligossacarides and amylose. Additionally, the glucoamylase turnover number (K_{cat}) on starch agrees with the values reported for *Aspergillus*, *Humicola,* and related species. For *Humicola* sp., Riaz *et al.* (2007) reported a K_{cat} value of 69 s^{-1}; for *Aspergillus niger*, a substantially higher K_{cat} value (343 s^{-1}) was reported (Riaz *et al.,* 2012). This study's K_m and K_{cat} values were superior to those reported for *Tetracladium* sp., 4.5 mg/mL and 0.75 s^{-1}, respectively (Carrasco *et al.,* 2017); for *Paecilomyces variotii*, 3.8 mg/mL and 41.7 s^{-1}, respectively (Michelin *et al.,* 2008). The catalytic efficiency displayed by this glucoamylase indicated how well it can catalyze a chemical reaction. The ability of the enzyme to lower the activation energy barrier describes its capacity to speed up a reaction while using a low amount of the enzyme (Robinson, 2015; Mbira, 2024).

CONCLUSION

Bioprospecting for enzymes from the environment has proved to be a reliable concept at producing novel enzymes that are suitable for the bio-economy. *Leohumicola incrustata* is one of many fungi that have a great potential to be included for the production of industrial enzymes. Glucoamylase is important in the manufacturing of syrup and bioethanol, its demand in industries has continued to rise. Therefore, this study showed that enzyme from *L. incrustata* exhibits promising catalytic property and this makes it an attractive candidate for industrial use.

Acknowledgements

Isolates credit: Dr. Christine Bizabani, Rhodes University, Mycorrhizal Research Laboratory, Grahamstown, South Africa.

Competing interests

The authors declare that they have no competing interests.

Data availability

Fungus used for this study was cultured from roots of ericaceous plants (*Erica chamissonis*) (Bizabani 2015). *Leohumicola incrustata* (Isolate code ChemRU330/Genbank Accession Number MF374380/The South African National Collection of Fungi Accession Number PPRI 17268), was obtained from Mycorrhizal Research Laboratory, Rhodes University, Grahamstown.

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