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Isolation of Hydrolytic Bacteria from Black Glutinous Rice Wash Water

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Abstract: *Natural sources for hydrolytic enzymes produced by bacteria in demand in industry were increased. Hydrolytic bacteria can be found in agricultural and household waste, like black glutinous wash water. This study aims to identify and evaluate the presence and hydrolytic potential of bacteria in washing waste from fermented black glutinous rice. Fermented black glutinous rice washing water was used as the sample for bacterial isolation. The obtained isolates were screened for hydrolytic activity using modified Bushnell–Haas Agar supplemented with specific substrates to assess cellulolytic, amylolytic, and pectinolytic activities. Hydrolytic activity was expressed as a hydrolytic index to determine the most potent isolate, which was subsequently identified at the molecular level. A total of eight bacterial isolates were successfully obtained, among which one isolate, designated NKH4, showed the highest hydrolytic potential based on screening results. Molecular identification revealed that NKH4 is closely related to Bacillus tequilensis, with a similarity value of 97.96%. This species has previously been reported to produce various hydrolytic enzymes. The selected isolate NKH4 exhibited a qualitative hydrolytic index of 0.89, demonstrating its ability to hydrolyze starch, cellulose, and pectin. Therefore, NKH4, identified as Bacillus tequilensis, holds strong potential as a producer of hydrolytic enzymes and may serve as a promising candidate for further biotechnological applications.*

Keywords: *Bacillus tequilensis; Black glutinous rice wash water; Enzyme screening; Hydrolytic bacteria; Hydrolytic index*

1. INTRODUCTION

The growing demand for natural products associated with the bioconversion process has led to an increase in research focused on the production and development of bacterial enzymes (Choi et al., 2015). Bacteria have long been recognized for their role in various natural transformation processes occurring in the environment. They produce enzymes that function as biocatalysts, facilitating a wide range of biochemical reactions. These enzymes can be isolated and utilized for different industrial and biotechnological applications (Adeoyo, 2025).

Bacteria are commonly selected as enzyme producers because they are easy to cultivate, grow rapidly, and require more efficient costs to maintain. In addition, bacterial systems often provide high enzyme yields, making them suitable for large-scale production. The global enzyme industry shows this increasing demand, with a market value estimated at USD 7.42 billion. It is expected to continue growing at an average annual rate of 6.3% from 2024 to 2030 (Adeoyo, 2025).

Hydrolytic enzymes, also known as hydrolases, have been utilized as a catalyst that facilitates the substrate breakdown through hydrolytic reactions involving hydrogen bonds. These enzymes are widely produced, particularly by bacteria. Their primary function is to degrade complex macromolecules into simpler compounds, making them highly valuable in bioconversion processes. Hydrolytic enzymes are mainly and often applied across multiple industries, including food and beverages, animal

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feed, agriculture, biofuel, textiles, and paper manufacturing as waste processing and by-products generating components (Shukla et al., 2022).

Hydrolase-producing bacteria are distributed across diverse environments such as soil, aquatic habitats, living organisms, and waste materials. Previous studies have identified cellulolytic bacteria, as a group of hydrolytic bacteria, in rose apple fruit, with Pseudomonas putida reported as one of the isolates (Prasetya et al., 2025). Thermophilic hydrolytic bacteria belonging to the genus Bacillus have also been isolated from hot springs in Saudi Arabia and demonstrated potential for amylase and protease production (Alrumman et al., 2018). Furthermore, hydrolytic bacteria have been detected in the rhizosphere soil of durian trees, where 52 bacterial isolates were identified with varying capacities to produce cellulase, amylase, protease, and lipase enzymes (Ni'matuzahroh et al., 2024).

Hydrolytic bacteria can be isolated from various waste materials and underutilized byproducts that serve as rich nutrient substrates for microbial growth. Previous studies have reported the isolation of hydrolytic bacteria from dried wheat sprouts, soybean pellets, sunflower meal, and cheese whey, all of which are agroindustrial byproducts. The identified bacteria belonged to diverse genera, including Serratia, Lactococcus, Enterococcus, Bacillus, Stenotrophomonas, Klebsiella, and Escherichia (Mazzucotelli et al., 2013).

In addition, an amylolytic bacterium identified as Bacillus macquariensis was isolated from cassava peel waste. The production of α-amylase was evaluated from the cell-free supernatant, yielding an activity of 7.58 U/mg protein. Following enzyme purification, the specific activity increased to 21.9 U/mg protein, indicating enhanced enzymatic efficiency (Bamigbade et al., 2014). Furthermore, proteolytic bacteria have been isolated from tofu production wastewater, where 28 distinct bacterial isolates with proteolytic potential were reported (Asril et al., 2019).

Rice washing water is a household and home industry waste that has not been widely utilized. Generally, the water from various types of rice is used as a fertilizer or biofertilizer substrate. Black glutinous rice is one type of rice often used as an alternative food and fermented tape food business (Saihani et al., 2015). Based on research, it is known that black glutinous rice washing water contains nitrogen, phosphorus, and potassium. It is also reported that the protein and starch content in black glutinous rice is 63% and 87%, respectively. Other research has also found that black glutinous rice washing water is suitable as an alternative medium for microbial growth. Several of these findings indicate the potential for the presence of indigenous bacteria in black glutinous rice washing water (Billa & Rahmayanti, 2023; Sulfianti et al., 2021; Zulkoni, 2014).

Research on the search or bioprospection of hydrolytic bacteria has been conducted previously. Still, bioprospecting hydrolytic bacteria derived from black glutinous rice washing waste has never been conducted before, making this the first study. Previous research related to the isolation of bacteria from black glutinous rice washing water has been conducted, but to isolate lactic acid bacteria. This research was conducted by Fusvita et al., 2018, a type of bacteria was obtained, which was identified as Enterococcus faecium from phenotypic tests. Research on hydrolytic bacteria was conducted on red rice water. Researchers discovered lactic acid bacteria (LAB) that also possess amylolytic and proteolytic activity. Eight out of ten LAB isolates were found to have amylolytic activity, and seven isolates had proteolytic activity (Adnan et al., 2017). Based on these explanations, this research aims to determine the presence and potential of hydrolytic bacteria in fermented black glutinous rice washing waste.

2. RESEARCH METHOD

Research Location and Time

This study was observational. The operational definition of variables in the screening and characterization assays includes the bacterial isolates as the independent variable. The dependent variables are represented by the observed characteristics of bacterial growth and the resulting hydrolytic index. Meanwhile, controlled variables consist of the type and condition of the culture media, as well as the incubation temperature and duration, which are maintained constant throughout the experiment. The research activities were conducted from

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August to December 2025. The entire process, from sample handling, enzyme production testing, to identification, was carried out in the Microbiology and Molecular Biology Laboratory, Faculty of Health Sciences, Maarif Hasyim Latif University. The DNA amplicon sequencing process was carried out with the assistance of PT. Genetika Science Indonesia for analysis at 1st BASE DNA Sequencing, Malaysia.

Preparation Fermented Black Glutinous Rice Wash Water

Black glutinous rice (250 g) obtained from traditional markets was washed with sterile distilled water. The washing water is not discarded; it is collected in sterile glass jars and sealed. The rice washing water is left to stand overnight to obtain the fermentation results.

Bacterial Isolation from Fermented Black Glutinous Rice Wash Water

Nutrient agar media (Merck) was prepared, and its pH was adjusted. The fermented rice washing water was then diluted 7 times using the serial dilution method, using 1 ml of the sample in 9 ml of 0.9% NaCl solvent. 0.1 ml of each dilution was then transferred to a Petri dish. The NA media was poured using the pour plate method, and the results were incubated in an incubator at 30 °C for 24 hours (Abidin et al., 2024). The isolated results were then characterized morphologically to distinguish their shape, edges, surfaces, elevations, textures, colors, and optical characteristics. The characterization results were written in the form of an isolate code, and then purification was carried out on new media. Characterization then continued to observe the cell morphology.

Isolate Preparation for Screening

Each isolate obtained was taken in one full loop, then inoculated into Nutrient agar media and incubated for 24 hours. After growth, a 0.9% (w/v) NaCl solution was prepared to suspend the isolate. The isolate was suspended, and its turbidity was compared with the McFarland standard turbidity of 0.5 (which is equivalent to 1.5×10^8. This standardization step ensured consistency in bacterial concentration for subsequent analyses.

Screening of Cellulolytic Bacteria Isolate

Screening of cellulolytic activity was carried out by placing a Whatman filter paper disc No. 1 (6 mm) containing the isolate suspension on Bushnell-Haas agar (BHA) medium, which was prepared with ingredients according to Nazar et al., (2018) in the form of (g/L) agar, K_2HPO_4 1; KH_2PO_4 1; $FeCl_3.6H_2O$ 0.05; NH_4NO_3 1; $MgSO_4.7H_2O$ 0.2; and $CaCl_2$ 0.02, then added 1% (w/v) carboxymethyl cellulose (CMC) as cellulose substrate. Furthermore, the isolates were incubated for 48 hours at 32 ºC. Observation of the results was carried out by pouring Congo red on the media to facilitate visualization of the clear zone, making it easy to measure.

Screening of Amylolytic Bacteria Isolate

Screening of Amylolytic activity was carried out by placing a Whatman filter paper disc containing the isolate suspension on Bushnell-Haas agar (BHA) medium according to Nazar et al., (2018) containing 1% (w/v) of starch as amylum substrate. Then, the dishes were incubated for 24 hours at 32 ºC. Observation of the results was carried out by pouring iodine, which forms a clear fluorescent zone on dark blue media, if the results were positive.

Screening of Pectinolytic Bacteria Isolate

Bushnell-Haas agar (BHA) medium modified by adding 1% (w/v) of pectin was prepared, and then the Whatman paper infused with the isolate was placed on the top of the medium. Then, the dishes were incubated for 48 hours at 32 ºC. Observation of the results was carried out by pouring Congo red, which helps to visualize the clear zone on dark blue media, if the results were positive.

Measuring the Clear Zone and Determining Hydrolytic Index

Clear zone measurements are carried out by subtracting the average of the vertical and horizontal measurements of the total diameter (clear zone and colonies) from the average horizontal and vertical colony diameter. Every result is then entered into the hydrolytic index (HI) formula to determine its hydrolytic capacity. Negative results are also included in the formula, because it is an average of four results. Hydrolytic

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Index formulas can be seen in the following equation (1) by Ni'matuzahroh et al. (2025).

$$Hydrolytic\ Index\ (HI) = \frac{Clear\ Zone\ Diameter - Colony\ Diameter}{Colony\ Diameter} \quad (1)$$

Identification of Potential Hydrolytic Bacterial Isolates

One bacterial isolate with the highest hydrolytic potential was biochemically characterized and molecularly identified using 16S rDNA primers 27F and 1492R. Extraction of DNA was carried out using TIANamp Genomic DNA Kit (TIANGEN inc) and followed their protocol until a supernatant containing DNA template was obtained for amplification. PCR amplification was performed after obtaining DNA purity as measured using an Å260/Å280 spectrophotometer (Genesys 10S UV Vis Thermo Scientific), with results of around 1.8 - 2.0, which stated that the template DNA was pure from contaminants (Nzilibili et al., 2018; Suryani et al., 2023).

The PCR reaction (Bio-Rad PCR) was prepared in a total volume of 50 µL, consisting of 25 µL GoTaq Green Master Mix, 5 µL of primers 27F (aga gtt tga tcc tgg ctc ag) and 1492R (ggt tac ctt gtt acg act t) (IDT Oligo), 5 µL of DNA template, and 10 µL of ddH_2O. The reaction mixture was subsequently subjected to amplification in a thermal cycler under the following conditions: initial denaturation at 94 ºC for 5 minutes; denaturation at 94 ºC for 30 seconds; annealing at 60 ºC for 30 seconds; extension at 72 ºC for 1.5 minutes; and final extension at 72 ºC for 10 minutes. The amplified product was then visualized by electrophoresis on a 0.8 % (w/v) agarose gel (Suryani et al., 2023).

The amplicons were sequenced at 1st BASE DNA Sequencing (Malaysia). The resulting sequence data were assembled into contigs using BioEdit software (version 7.2). Subsequently, the sequences were compared with those available in the NCBI database using the BLAST algorithm (accessible at https://blast.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using MEGA software (version 11), and the phylogenetic tree was constructed based on the Neighbor-Joining method with 1,000 bootstrap replicates.

Data analysis

The data obtained, including bacterial characteristics, hydrolytic index (HI) values from screening tests for each hydrolytic enzyme, identification results, and phylogenetic tree analysis, were analyzed descriptively. The results were presented in tables and graphs. Based on the data analysis, a discussion was conducted in accordance with the findings obtained.

3. RESULTS AND DISCUSSION

Results

The isolation process yielded eight bacterial colonies with varying characteristics, reflecting differences in colony morphology and cell morphology commonly observed in environmental samples. One bacterial colony, coded NKH2, was found embedded within the medium, making it difficult to purify. The eight colonies originated from different plates and dilution levels, indicating a diverse microbial population. Table 1 shows a variety of colony characteristics, while Table 2 shows two Gram-positive and five Gram-negative bacteria.

The seven purified isolates were then screened for hydrolytic activity using BHA with various specific substrates. Based on screening test results for amylase, cellulase, and pectinase, one bacterial isolate, NKH4, exhibited the expected amylolytic, pectinolytic, and cellulolytic activity, whereas the other isolates showed varying levels of activity. Screening was carried out qualitatively by observing the clear zones formed in the media. The results show that only one isolate, NKH4, exhibits three hydrolytic activities, with a total hydrolytic index of 0.89 (Figure 1B, F, and J). NKH1 and NKH5 isolates showed only cellulolytic activity with a lower hydrolytic index than NKH4 (Figure 1E and F). The screening results are presented in Tables 3 and Figure 1.

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Table 1. Colony morphology characteristics of black glutinous rice washing water bacterial isolates

Isolate	NKH1	NKH2	NKH3	NKH4	NKH5	NKH6	NKH7	NKH8
Shape	Circuler	Spindel	Irreguler	Irreguler	Circuler	Circuler	Irreguler	Circuler
Margin	Entire	Entire	Erose	Lobate	Erose	Erose	Entire	Entire
Elevation	Convex	Flat	Raised	Raised	Flat	Flat	Convex	Convex
Surface	Glistening	Smooth	Smooth	Smooth	Foggy	Glistening	Smooth	Glistening
Optic	Opaque	Opaque	Opaque	Opaque	Transluscent	Opaque	Opaque	Opaque
Pigment	Yellowish white	White	White	White	White	White	White	White
Location	Surface	Sub-Surface	Surface	Surface	Surface	Surface	Surface	Surface

Potential bacterial isolates were also tested for biochemical characteristics to determine their phenotypic traits using the IMVIC test, which consists of indole, MR, VP, and citrate assays. Furthermore, all bacterial isolates were evaluated for their catalase production and carbohydrate fermentation capabilities to provide additional insight into their metabolic profiles. The test results for each isolate varied and are presented in Table 3.

Table 2. Cell morphology characteristics of black glutinous rice washing water bacterial isolates

Isolate	NKH1	NKH2	NKH3	NKH4	NKH5	NKH6	NKH7	NKH8
Shape	Bacilli	-	Bacilli	Bacilli	Cocci	Bacilli	Bacilli	Coccobacilli
Structure	Mono	-	Mono	Mono	Staphy	Diplo	Diplo	Mono
Gram	Negative	-	Negative	Positive	Positive	Negative	Negative	Negative
Endospore	Negative	-	Negative	Positive	Negative	Negative	Negative	Negative

Table 3. Hydrolytic activity of black glutinous rice washing water bacterial isolates based on the hydrolytic index

	NKH1	NKH3	NKH4	NKH5	NKH6	NKH7	NKH8
Amylolytic	-	-	0.4	-	-	-	-
Cellulolytic	0.37	-	1.13	0.18	-	-	-
Pectinolytic	-	-	1.16	-	-	-	-
Average	0.12	-	0.89	0.06	-	-	-

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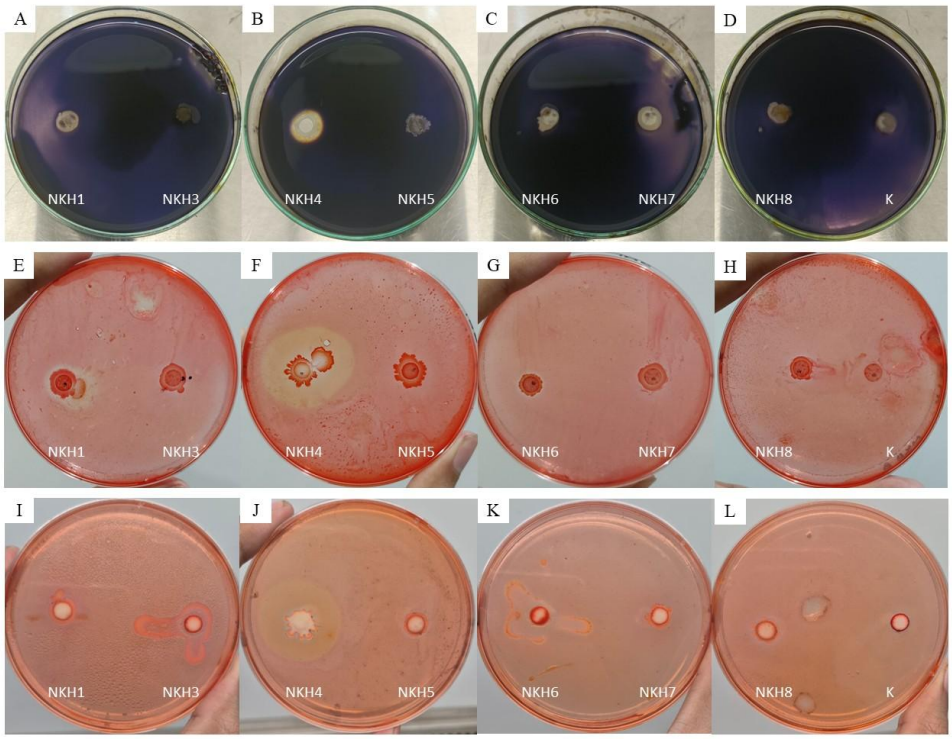


Figure 1. Hydrolytic screening test of fermented black glutinous rice wash water; **A-D**. Amylolytic assay; **E-H**. Cellulolytic assay; **I-L**. Pectinolytic assay

One isolate known to have the highest average hydrolytic index was identified molecularly. The DNA extraction results showed a purity of 1.85 and a quantity of 350, indicating that the DNA was sufficient for amplification and sequencing. The results of sequencing and analysis indicated that the NKH4 bacterium is a type of *Bacillus tequilensis* with 97,96% similarity. Phylogenetic analysis showed that the NKH4 isolate grouped in a clade with several strains of *Bacillus tequilensis* and was separate from other species in the *Bacillus subtilis* complex. The bootstrap value of ±82% indicated a sufficient level of confidence in the grouping. The results can be seen in Figure 2.

Table 4. Biochemical characterization results of NKH4

Characters	NKH4
Indole	-
MR	-
VP	+
Citrate utilization	+
Catalase	+
Carbohydrate fermentation	
Glucose	-
Lactose	-
Sucrose	+ (g)

Gas: gas production

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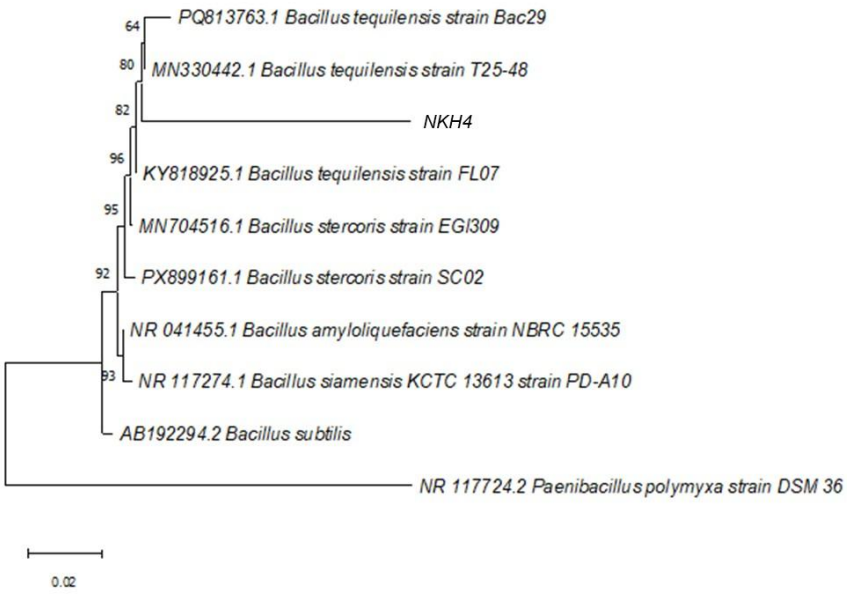


Figure 2. Phylogenetic tree of the NKH4 isolate

Discussion

Based on Table 1, several bacterial isolates with varying characteristics were found, indicating that black glutinous rice washing wastewater contains bacteria. The common and dominant bacteria in rice washing water are generally from the Proteobacteria, Cyanobacteria, Firmicutes, and Bacteroides groups (Chen et al., 2021). The bacteria found in black glutinous rice washing water may originate from the black glutinous rice grains. Glutinous rice grains have been reported to harbor endophytic bacteria, although their diversity is low. Bacteria attached to black glutinous rice grains are also suspected to originate from contamination during storage by air (Laca et al., 2006; Prasetya et al., 2025). The differences in morphology of all isolated isolates may occur due to bacterial responses to environmental conditions (Yang et al., 2016).

The screening results of the seven bacterial isolates showed that only one isolate possessed amylolytic, cellulolytic, and pectinolytic hydrolytic activities. Previous studies on rice endophytic bacteria found amylolytic, pectinolytic, and cellulolytic activities. These three enzymes are thought to play a role in the endophytic processes of bacteria. Hydrolytic bacteria, such as proteolytic bacteria, can be found in the soil and various agricultural byproducts (Elbeltagy et al., 2000; Mano & Morisaki, 2008; Mazzucotelli et al., 2013; Salsabilla et al., 2024; Saputri et al., 2023).

More than one type of bacterium exhibiting cellulolytic activity was identified, likely because several isolates originated from the husk of black glutinous rice that had adhered to the grains. Rice straw and husk are known to contain approximately 35% cellulose of the total husk weight. Some bacteria are presumed to persist on the grains despite milling processes intended to remove the black glutinous rice husk. Microorganisms associated with the husk may derive from endophytic microbes that migrate from the root system by degrading cellulose in the cell walls throughout different parts of the black glutinous rice plant (Hardoim et al., 2015; Osman & Abd El-Galeel, 2008; Yunus et al., 2019).

Black glutinous rice contains a substantial amount of starch in the form of amylopectin in grains after milling, accounting for approximately 70% of the total weight, whereas amylose constitutes about 13%. In addition, black glutinous rice contains approximately 11% protein (Mustofa et al., 2024). These nutritional components are expected to serve as specific substrates for the three enzymatic reactions. However, the results showed that only one type of bacterium exhibited hydrolytic activity. This may

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be attributed to the absence of genes encoding the synthesis of the relevant enzymes in several bacterial isolates (Urvoy et al., 2021). Furthermore, antinutritional factors (ANFs) commonly present in rice may inhibit enzymatic hydrolysis. Typical ANFs found in rice include saponins, polyphenols, and phytic acid (Samtiya et al., 2020; Wordu et al., 2021). It is also suggested that these ANFs in the rice grains may be leached along with other nutrients, such as protein, crude fiber, and lipids (Abba et al., 2021).

Based on molecular identification (Figure 2), the superior hydrolytic isolate was identified as *Bacillus tequilensis*. Members of the genus *Bacillus* are Gram-positive, endospore-forming bacteria commonly found in aquatic and soil environments. Previous studies have also reported the frequent isolation of *Bacillus* species from agricultural waste. The *Bacillus tequilensis* identified in this study exhibited large and irregular colony morphology, consistent with earlier reports. However, several differences in biochemical characteristics were observed when compared to previous studies. For instance, indole production, which was reported as positive in earlier studies, was found to be negative in the present study. This discrepancy may be attributed to strain-level variation within the species. Indeed, previous studies have demonstrated that *Bacillus tequilensis* strains EGB3 and A1C1 exhibit distinct biochemical profiles (Ghosh & Chatterjee, 2020; Kapil et al., 2021).

Bacillus species capable of producing polyhydroxyalkanoates (PHA) have been isolated from leaf litter. Other studies have also reported the isolation of cellulolytic *Bacillus* from compost, soil, and livestock sludge (Kim et al., 2012; Zaki, 2018). Members of the genus *Bacillus* are well known for their wide range of applications in both agricultural and industrial sectors. In agriculture, *Bacillus* species are widely utilized as nitrogen-fixing bacteria, nutrient solubilizers, siderophore producers, and biocontrol agents. In addition, several *Bacillus* species are recognized for their ability to produce extracellular enzymes; for instance, *Bacillus licheniformis* is known to produce amylase and protease enzymes (Dumitru & Habeanu, 2021; Tiwari et al., 2019).

The ability of *Bacillus tequilensis* to produce hydrolytic enzymes has been widely reported in previous studies. Ghosh and Chatterjee (2020) demonstrated that *Bacillus tequilensis* isolated from the gut of *Perionyx excavatus* exhibited starch (amylase) hydrolytic activity. In another study, *Bacillus tequilensis* UTMSA14, isolated from geothermal hot springs in Indonesia, was reported to produce cellulase and protease (Fachrial et al., 2020). Similarly, *Bacillus tequilensis* G9, isolated from African snails, showed cellulolytic activity (Dar et al., 2019). Furthermore, a strain of *Bacillus tequilensis* isolated from tobacco wrapper leaves in China was found to produce pectinase (Zhang et al., 2019).

4. CONCLUSION

A total of three hydrolytic bacterial isolates were obtained from the fermentation of black glutinous rice washing water out of eight isolates, with NKH4 identified as the superior isolate. The superior isolate was determined based on the highest hydrolytic index, with a value of 0.89. Isolate NKH4 demonstrated the ability to hydrolyze pectin, cellulose, and starch, whereas the other isolates, NKH1 and NKH5, were only capable of cellulose hydrolysis. Morphologically, the superior isolate NKH4 exhibited irregular and lobate colony forms, appeared white in color, and was identified as Gram-positive rod-shaped bacteria with endospores. Biochemical characterization using the IMViC test showed positive results for the VP test and citrate utilization, while carbohydrate fermentation tests indicated positive sucrose fermentation with gas production, along with a positive catalase reaction. Molecular identification revealed that the superior hydrolytic isolate NKH4 was closely related to *Bacillus tequilensis* with a similarity of 97.96%. This study may serve as a basis for further bioprospecting research aimed at developing the enzymatic activity of the identified *Bacillus tequilensis* NKH4 on black glutinous rice wash water as a specific substrate. Further research is required to conduct quantitative analyses of hydrolytic enzyme production and to optimize production conditions, in addition to investigating the potential utilization of various agricultural waste materials as alternative substrates.

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