



AMYLOLYTIC POTENTIAL OF *Arthrobacter* sp. AND *Bacillus aerius* FROM WASTE DUMP-SITES OF TWO BAKERIES LOCATED WITHIN KADUNA METROPOLIS, NIGERIA

*Tonidunni Ibukunola Obafunmi, Garba Faiza Bakori and Yunus Sumayya Lawal

Department of Applied Biology, College of Science and Technology, School of Applied Science, Kaduna Polytechnic, PMB 2021, Kaduna, Nigeria.

*Corresponding authors' email: otibukunola@kadunapolytechnic.edu.ng Phone: +44(0)7766162585

ABSTRACT

Bakery waste dumpsites are believed to harbour a variety of bacteria due to the presence of organic materials like flour, sugar, and other food residues. The conditions in these environments support the growth of bacteria with the ability to degrade starch into simple sugars because they produce amylases, an enzyme pivotal to the food and beverage production. As Amylases progressively gain relevance in several other starch-based processes, their availability becomes insufficient, and the need for amylases with unique and superior efficiency increases. The focus of this study was to isolate and characterize bacteria with amylase activity from the soil of bakery waste dumpsites to provide valuable insight into cheap sources of amylolytic bacteria and their potential biotechnological applications. The starch-agar medium was used to isolate and screen amylase-producing bacteria by streak plate method. Two isolates, A2 and A4, showing the most hydrolytic activities of 21 ± 0.1 mm and 25 ± 0.1 mm, respectively, were identified by their cultural and cellular morphologies, biochemical characteristics, 16S rRNA gene sequence, and nearest phylogenetic neighbour as *Arthrobacter* sp. and *Bacillus aerius*. Based on their culture and amylolytic temperatures, *Arthrobacter* sp. (37°C) and *Bacillus aerius* (45°C) tend to produce amylases that can be explored in low to moderate and moderate to torrid industrial processes, respectively.

Keywords: Bacteria, Bakery Waste, 16S rDNA Sequencing, Amylases, Applications

INTRODUCTION

A drift away from the use of inorganic or synthetic substances in catalytic processes is driven by the need for a sustainable environment and the diverse industrial niche occupied by enzymes considering their biodegradability, innate catalytic attribute, high efficiency, stability under varying reaction conditions, and specificity (Sundarram & Murthy, 2014; Sindhu et al., 2018; Mondal et al., 2022). Microbial enzymes are most employed because the cultivation of microorganisms is cost-effective, and they produce genetically modifiable enzymes with better reproducibility and reliability than those of animal and plant origins (Burhan et al., 2003; Sindhu et al., 2018). One of such top thriving enzymes vying for global market share is amylase (Sundarram & Murthy, 2014; Mondal et al., 2022), which converts polysaccharides to simple sugars by glycosidic bond cleavage of starch molecules or their derivatives (oligosaccharides) (Taniguchi & Honnda, 2009). Amylases have a wide array of uses, including the production of starch (Nielsen & Borchert, 2000; Duan et al., 2021), textiles (Montazer & Harifi, 2018), detergents (Nielsen & Borchert, 2000), beverages (Santos et al., 2023; Xiang et al., 2023), biofuels (De Castro et al., 2011), food processing (Sindhu et al., 2018; Adroit Marker Research, 2019) and medicals (Azzopardi et al., 2016).

Current interest in amylases focuses on increasing the yield, extending substrate utilization and stability of the enzyme at extreme temperatures and pH levels required for commercial processes, and reducing the cost of industrial starch degradation process (Bozic et al., 2011; Far et al., 2020; Mondal et al., 2022). Recombinant technology and extremophiles producing hyperthermostable amylolytic enzymes are being explored as potential solutions to these problems (Nielsen & Borchert, 2000; Mondal et al., 2022). The quest for more process compatibility rises as the applications of amylases widen (Pandey et al., 2000); inferably, the expedition of competent amylolytic microorganisms has also become endless. Of all microbial

sources available for amylase production, bacteria have the most capability for large-scale production (Far et al., 2020), most significantly the thermostable *Bacillus* strain garnering about 60% of commercial enzymes on sale.

Common groups of amylases sourced from some bacteria include α -amylase, with an increasing market worth projected at US \$465.5 million in a decade (Adroit Marker Research, 2019), sourced from *Bacillus licheniformis* (Kandra, 2003; Bozic et al., 2011; Torabizadeh et al., 2014), *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus* (Sindhu et al., 2018), *B. cereus* (Wang et al., 2020), and *Geobacillus thermoleovorans* (Mondal et al., 2022), β -amylase first produced from *Bacillus megaterium* (Mondal et al., 2022) and subsequently from *Bacillus cereus*, *Bacillus polymyxa* (Niziolek, 1997), and *Pseudomonas* spp. (Karki, 2020), debranching enzymes (oligo-1,6-glucosidase, pullulanase, amylo-1,6-glucosidase, and isoamylase) from *Clostridium*, *Bacillus*, *Thermoanaerobacter*, *Thermobacteroides* spp., and *Pseudomonas amyloclavata* (Mondal et al., 2022), glucoamylase (γ -amylase) from *Bacillus amyloliquefaciens* (Abd-Elhalem et al., 2015; Wang et al., 2020), *Paenibacillus amylolyticus*, *Bacillus* sp., *Clostridium* spp., *Flavobacterium* sp. (Lincoln et al., 2019), *B. subtilis*, *B. cereus* (Wang et al., 2020), and α -glucosidase from *Lactococcus lactis* (Ikegaya et al., 2022).

The study of Bozic et al. (2011) has suggested a decrease in starch conversion to a single step using thermostable γ -amylase as a cost-cutting strategy. Additionally, investigations are ongoing into glucoamylase derived from archaea (Prakash et al., 2009) because glucoamylase is the only enzyme capable of wholly breaking down starch into glucose (Mondal et al., 2022), and the fungal glucoamylase employed in the second step (Sundarram & Murthy, 2014) i.e., in conversion of limit dextrin to glucose is limited by its optimal temperature of 60°C (Bozic et al., 2011). The selection of an appropriate microorganism is therefore a key determinant in the synthesis of targeted enzymes (John,

2017); more so, scouting and screening of bacteria with high amylase activity might facilitate the development of novel amylases required in industrial processes (Gopinath et al., 2017; Okunwaye et al., 2021; Niyomukiza et al., 2023). This present study aims to explore bakery waste for amylolytic bacteria, characterize the bacterial isolate using biochemical tests and molecular techniques, and highlight their potential field of application.

MATERIALS AND METHOD

Sample Collection

Soil samples mixed with wastes from two waste dump sites of Tony's and Swiss bakeries located correspondingly at Television and Sabon-tasha within Kaduna metropolis were collected aseptically into clean polythene bags and transported to the microbiology laboratory in the Department of Applied Biology, Kaduna Polytechnic Tudun-wada main campus.

Isolation of Bacteria from Soil and Bakery Waste Samples and Screening for Amylase Activity

Alpha-amylase can break down starch into oligosaccharides (maltose, maltotriose, glucose, and limit dextrin). Likewise, β -amylase and glucoamylase yield maltose or glucose while debranching and α -glucosidase liberate α -sugars and α -glucose respectively (Mondal et al., 2022). Based on the ability of bacterial amylases to hydrolyse starch and or glycogen, starch-agar medium composed of 1% starch and 2% agar, was used for selective isolation, detection, and screening of bacteria from the collected soil samples.

After serial dilution of the collected samples was done to thin out the microbial population (Willey et al., 2008; Gopinath et al., 2017), an aliquot of 0.1 ml each was drawn from 10^{-5} and 10^{-6} dilutions and inoculated following all standard procedures of the spread plate technique (Sinha, 2010). Each duplicate plate set was incubated separately at 37 °C and 45 °C for 24 hours. Bacterial colonies showing amylolytic activity by starch hydrolysis were sub-cultured on the starch-agar plates. After incubating at initial growth culture temperatures for 12 to 48 hours, a deep, blue-coloured starch and iodine complex was formed after sufficient amounts of Gram's iodine solution was added onto each of the separate plates. In contrast, no blue colour was formed in the clear degradation zone. These isolates were identified as amylolytic, and two, which displayed the maximum diameter of the hydrolytic zone, A2 and A4, were selected for identification (Gupta et al., 2003).

Morphological and Biochemical Characterization of Amylase Producing Bacterial Isolates from Soil Mixed with Bakery Wastes

Pure culture plates were observed, and the cultural characteristics of the colonies were described. The cellular morphology of the isolates was identified by gram staining and microscopy, and a few of their biochemical activities were characterized by standard biochemical tests as described by Bergey's Manual of Systematic Bacteriology.

Molecular Identification of Amylase-Producing Bacteria from Soil Mixed with Bakery Wastes

DNA Extraction

The Bioneer AccuPrep genomic DNA extraction kit was used to extract genomic DNA from the amylolytic A2 and A4 isolates. To obtain the bacterial cell pellet, the preparation was centrifugated at 10,000 x g for 300 seconds then washed with proteinase K (20 μ l) and RNase (10 μ l) to inactivate and remove proteins and RNA, respectively leaving only DNA;

this mixture was then incubated at room temperature for 2 minutes. Bacterial cell lysis was achieved on addition of GB lysis buffer (200 μ l), followed by vortexing and incubating at 60 °C for 10 minutes. After 400 μ l of ethanol (anhydrous) was introduced and adequately mixed using a pipette, the resulting lysate was carefully turned into the upper reservoir of a binding column and centrifuged for 60 seconds at 8,000 rpm. Two wash steps were performed using 500 μ l of W1 and W2 buffers, respectively, followed by centrifugation at 8,000 rpm for 60 seconds each time to get rid of contaminants and ascertain DNA purity. After discarding the solution in the collection tube, a final 13,000 rpm centrifugation for 1 minute was done to eliminate any remaining ethanol and droplets adhering to the tube. For the elution step, and 200 μ l of EA buffer was introduced into the affinity column tube placed in a sterile 1.5 ml tube, then incubated at 25°C for about 60 seconds, and lastly centrifuged at 8,000 rpm for 1 minute to collect the eluted DNA.

PCR Amplification of 16S rDNA from Genomic DNA of A2 and A4 Bacterial Isolates

Ribose-1 (5'-GGACTACAGGGTATCTAAT-3') and ribose-2 (5'-AGAGTTTGATCCTGG-3') of 16S rDNA were respectively designed as forward and reverse primers to amplify the 16S rRNA gene from the genomic DNA extracts. Amplification occurred in 25 thermocycles set at an initial denaturation temperature of 95 °C for 5 minutes, with denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute, and extension at 72 °C for 1 minute and a final extension at 72 °C for 5 minutes (Suchm, 2011).

16S rDNA Visualization and Quantification

The PCR product amplification was confirmed on 1% agarose gel electrophoresis at 80v for 45 minutes, and the DNA bands were visualised using the Bio-Rad gel imaging system.

DNA Sequencing

The PCR product was purified using the Bio-Rad PCR Kleen clean-up kit in a spin column following the manufacturer's instructions. After the Sanger sequencing of the purified PCR product, the sequence data were analysed through NCBI-Blastn (<http://blast.ncbi.nlm.nih.gov>) to match possible organisms.

Phylogenetic Tree and Evaluation

The Geneious, Java Version 1.8.0_60-b27 (64-bit) was used to create, evaluate, and confirm the identities of amylolytic isolates A2 and A4 by their phylogenetic relatedness by Neighbour-Joining (www.geneios.com).

RESULT AND DISCUSSION

Bacterial Isolates from Soil and Bakery Waste Samples and their Amylolytic Capability

This study isolated bacteria from a mixture of soil and bakery wastes. Their ability for amylase production was also determined by their visible growth and degradative activity on starch-agar medium. Inferably, amylolytic bacteria are naturally abundant in the soil because the heterogeneous nutrient in it helps sustain microbial life (Baath, 1998; Madhav et al., 2011). Moreover, bakery waste further enriches the soil with starch, which is a carbon source for amylase producers (Singh & Kumari, 2016). Colonies A2 and A4 displayed the best hydrolytic potential on solid starch-agar medium, giving zones of starch digestion of 21 ± 0.1 mm and 25 ± 0.1 mm, respectively (Table 1). It is presupposed that the A2 isolate is a mesophile while A4 is a thermophile because the optimum temperature of growth and amylase activity for

the A2 isolate was at 37 °C; on the other hand, the clear zone of hydrolysis measured by diameter (mm) increased at 45 °C incubation temperature for the A4 isolate and according to Angelin and Kavitha (2022), hydrolytic bacteria thriving at temperatures between 40 °C to 85 °C are thermophilic and often good producers of thermostable amylases.

Morphological and Biochemical Characteristics of Amylase Producing A2 and A4 Bacterial Isolates from Bakery Dumpsites

Due to similarities in the morphological and biochemical properties of the bacterial isolates A2 and A4 from bakery

waste dumps, both were thought to belong to the genus *Bacillus* (Table 1). This result and other reports on *Bacillus* spp. with amylase-producing ability from food wastes (Abd-Elhalem et al., 2015; Krishma & Radhathirumalaiarasu, 2017; Okunwaye et al., 2021), industrial waste (Sundarram & Murthy, 2014), plantation soils (Madhav et al., 2011; Lincoln et al., 2019), and soils of various garbage dumpsites (Madhav et al., 2011; Singh & Kumari, 2016) corroborate Pandey et al. (2000), who reiterated that *Bacillus* spp. is the most ubiquitous of all amylase-producing bacteria.

Table 1: Morphological and Biochemical Characteristics of Amylase Producing Bacterial Isolates from Soil and Bakery Waste

Characteristics		Isolates	
		A2	A4
Colonial Morphology	Form	Circular	Circular
	Pigmentation	Creamy	creamy
	Elevation	Flat	Flat
	Margin	Smooth	Smooth
Cellular Morphology	Form	Rod	Rod
	Arrangement	Pairs	Pairs
	Gram's reaction	Positive	Positive
Biochemical Activity	Catalase	Positive	Positive
	Oxidase	Negative	Negative
	Citrate	Positive	Positive
	MR	Negative	Negative
	VP	Positive	Positive
	Indole	Negative	Negative
	Starch hydrolysis	Positive	Positive
Incubation Temperature		37 °C	45 °C
Diameter of zone of starch hydrolysis (mm)		21 ± 0.1	25 ± 0.1

Molecular Characteristics of Amylase Producing A2 and A4 Bacterial Isolates from Soil and Bakery Waste

The amplified DNA band sizes were approximately 789 bp on agarose gel, as shown in Figure 1, while the sequence data generated was 516 bp for the A2 isolate and 517 bp for A4 isolate. The discrepancy in the gel and sequencing band sizes could result from a mixture of DNA fragments, contamination

in the gel, structural variations in the 16S rDNA region amplified, or sequence error; these scenarios were observed as limitations to the 16S rDNA sequencing. To enhance accuracy, a reduction in the number of thermal cycles and other methods, such as the minion nanopore and circular consensus broad sequencing, are being suggested (Patel et al., 2017; Johnson et al., 2019; Matsuo et al., 2021).

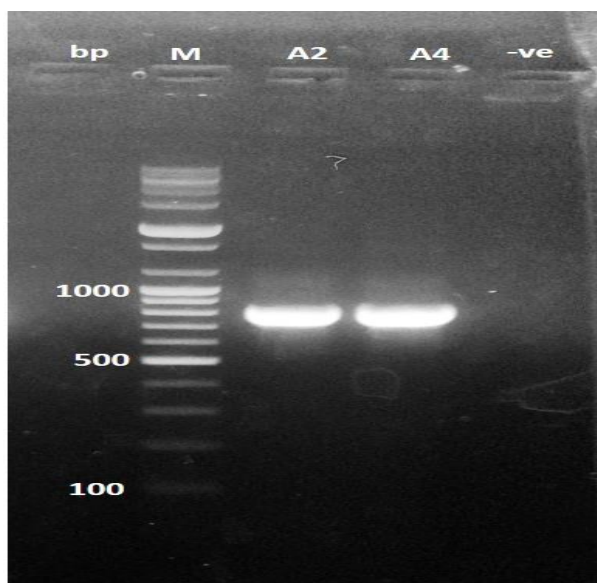


Figure 1: Bio-Rad Gel Imaging of Amplified 16S rDNA of Bacterial Isolates A2 and A4 from Soil and Bakery Waste Showing Band Sizes ~789bp. Lane; 1: Marker Sizes (bp), 2: DNA Marker, 3: A2 Band, 4: A4 Band, 5: Negative control

The most frequent sequence lengths compared with databases are 500 to 1,500 bp (Clarridge, 2004; Woo et al., 2008). However, Johnson et al. (2019) disputes the integrity of sequencing short DNA fragments of 16S rDNA and proposes whole-length sequencing (1,500bp) as the most reliable for the classification of bacteria to specie and or to strain level due to the inadequacy in the sub-regions of specific bacterial genera. Conversely, from a phylogenetic perspective, Clarridge (2004), in his review, was able to demonstrate insignificant differences in both sequence lengths for some bacteria and those with hypervariable regions in their first 500bp. Similarly, Matsuo et al. (2021) compared the full and partial length 16S rRNA gene results and reported a strong correlation in many bacteria genera studied.

Based on the aligned sequences, high query cover, and percentage nucleotide similarities of 85.55% and 87.82 with the respective matching organisms on the NCBI database, A2 isolates were identified as *Arthrobacter* sp. and A4

as *Bacillus aerophilus* (Table 2). The investigation of the identities of *Arthrobacter* sp. and *Bacillus aerophilus* by 16S rDNA sequencing reaffirms the advantage of the technique (Clarridge, 2004; Loong et al., 2016), as they were initially indistinguishable by their phenotypic attributes. The highly conserved and distinct variable regions in the 16S rRNA gene of bacteria, especially the bacilli genera, underpins the technique which has been extensively exploited in the identification of novel organisms from clinical samples (Clarridge, 2004; Woo et al., 2008; Patel et al., 2017; Johnson et al., 2019; Matsuo et al., 2021) and has also gained relevance in the classification of industrially significant amylolytic bacteria (Abd-Elhalem et al., 2015; Krishma & Radhathirumalaiarasu, 2017; Niyomukiza et al., 2023). Furthermore, the application of 16S rRNA gene analysis is paving new pathways for the emerging field of metagenomics (Nair et al., 2017; Nasir et al., 2020).

Table 2: Molecular Nomenclature of Amylolytic Bacterial Isolates from Bakery Waste Dumpsites

Isolate code	Proposed Identity of Organisms	Percentage Identity (%)	Matching Organism & NCBI Accession number
A2	<i>Arthrobacter</i> sp.	85.55	<i>Arthrobacter</i> sp. strain JI22 (MT126254.1)
A4	<i>Bacillus aerophilus</i>	87.82	<i>Bacillus aerophilus</i> strain Cg1 (MZ677267.1)

Phylogenetic Tree and Evaluation

The evolutionary relationship of *Arthrobacter* sp. and *Bacillus aerophilus* is represented in Figures 2 and 3, respectively. Over a 100% query cover, the *Arthrobacter* sp. A2 sequence shared 85.55% identity with *Glutamicibacter* spp., an unidentified bacterium, *Arthrobacter mysorens*, *Arthrobacter arilaitensis* and several strains of *Arthrobacter* sp., including the most closely related strain, *Arthrobacter* sp. JI22, with which it shared 60% ancestral homology. On the other hand, an evolutionary distance of 81% was obtained for *Bacillus aerophilus* A4 and *Bacillus aerophilus* strain Cg1 which gave the highest identical aligned sequence of 87.82% over a query cover of 99.81%. This *Bacillus aerophilus* A4 16S rDNA gene also shared common lineage with *Bacillus subtilis* strain MBF21 (87.7%), some strains of *Bacillus altitudinis* (87.7%),

Bacillus aerophilus (87.7%), *Bacillus pumilus* (87.7%) and 87.5% for *Bacillus statosphericus* and *Bacillus vallismortis*. Generally, organisms having >95% nucleotide sequence identity are assigned the same genus, whereas > 97% identical sequences are placed in the same species category (Abd-Elhalem et al., 2015; Johnson et al., 2019). Therefore, the similarity in this study is low and may be due to a narrow 16S rDNA sequence length employed. Hence, the required percentage identity for naming the A2 to specie level was insufficient, and the proposed species taxon for A4 is based on the propinquity of phylogeny and correlating phenotypic features. In consonance with Loong et al. (2016), Johnson et al. (2019), and Matsuo et al. (2021), the adequacy of 16S rRNA gene technology to classify some bacteria to specie level could be baulked by short read sequencing.

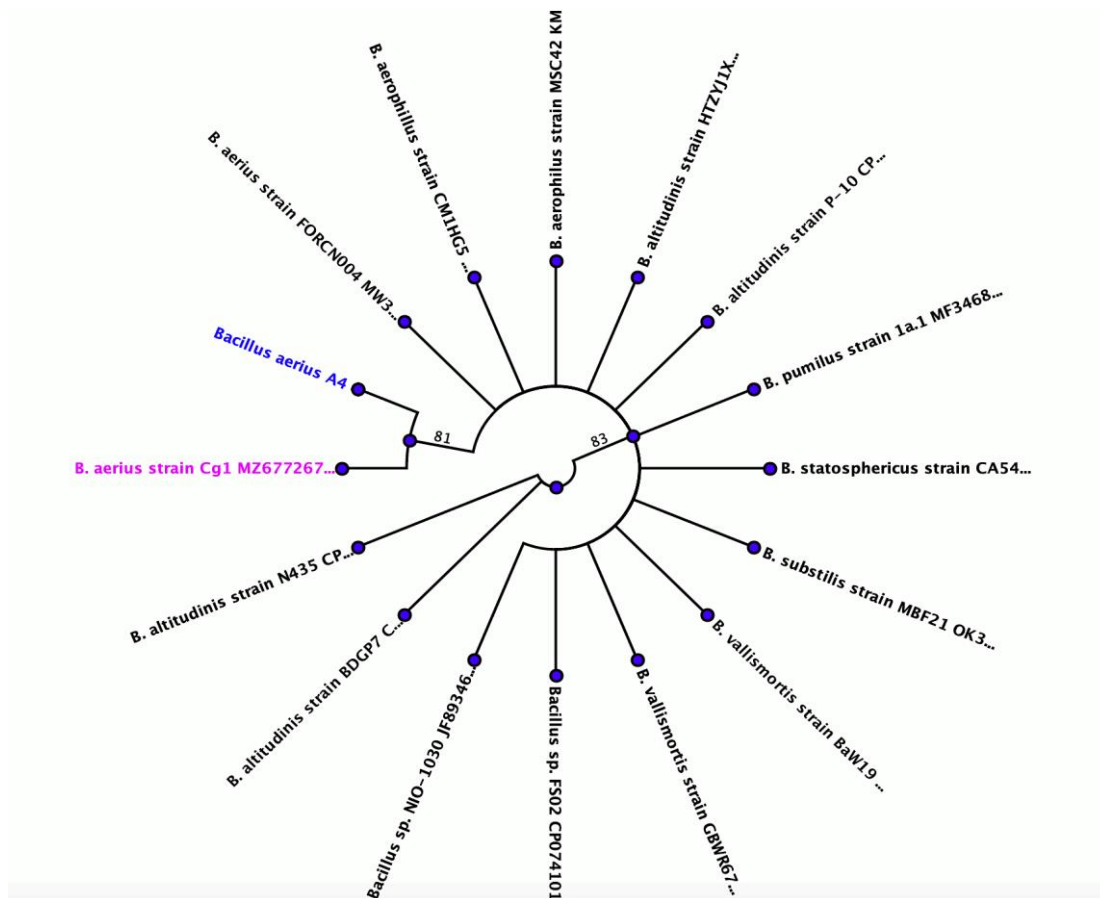
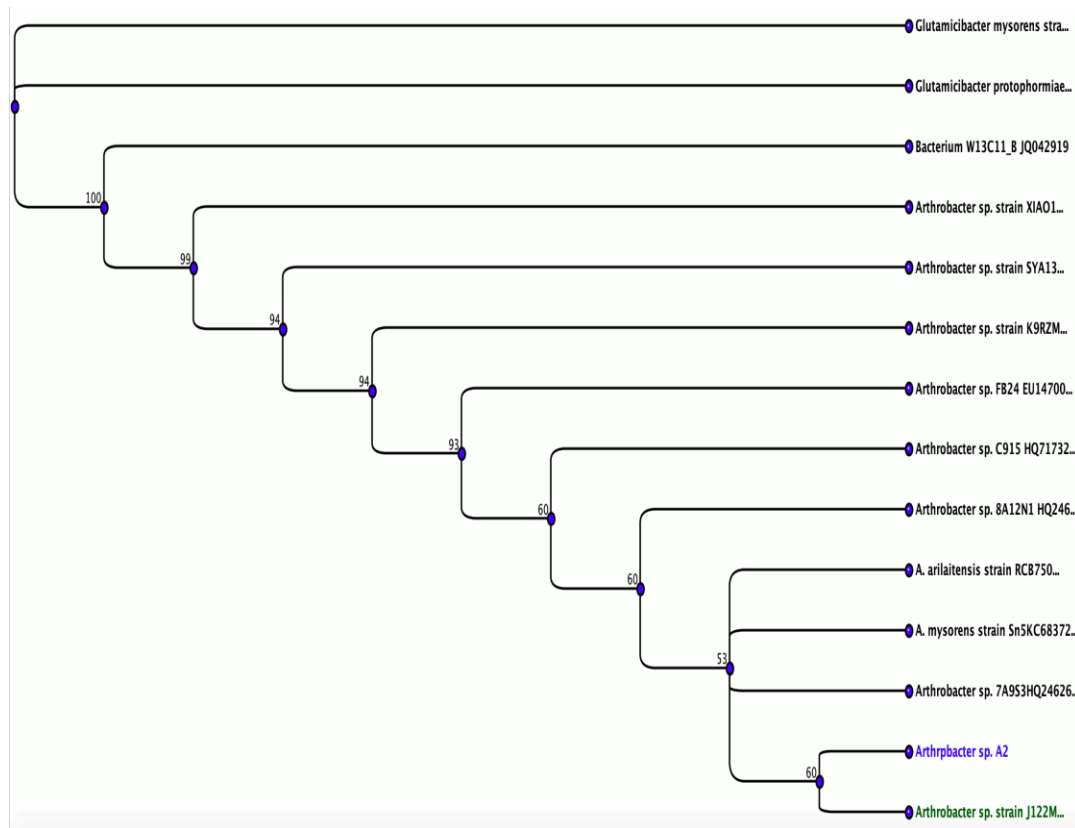


Figure 2: The Geneious Rooted Tree Layout of the Phylogenetic Tree by Neighbor-Joining of 16S rRNA Genes based on Aligned Sequences of *Arthrobacter* sp. A2 and 13 Database (NCBI) Comparable Bacteria. Nodes: Values (%) of Evolution, Blue: *Arthrobacter* sp. A2 from Soil and Bakery Wastes Green: Closely Related Genus

Possible Applications of Amylases from *Arthrobacter* sp. A2 and *Bacillus aerius* A4

Niyomukiza et al. (2023) demonstrated with *B. aerius* from food waste that, indeed, substrate concentration, temperature and pH are factors controlling the effect of amylase activity; the study also expressed the unique potential of *B. aerius* for maximum productivity in an acidic medium at 40 °C and average productivity at extensive pH range. In addition, the result of Ghazanfar et al. (2022) reiterated the report of Ganesan et al. (2020), which described the biofuel production suitability of a hot spring dwelling, temperature tolerant, high-yielding stable *Bacillus aerius* strain with a broad cellulose capacity to degrade polysaccharides into ethanol at 50 °C optimum temperature. The 45 °C optimum temperature for growth and amylolytic activity of *Bacillus aerius* A4 obtained in this study is in complete tandem with these reports, and according to Sundarram and Murthy (2014), the possibility of mesophilic contamination is curtailed using enzymes obtained from thermophiles. Such thermostable enzymes (α -amylases, β -amylase combined with pullulanase or glucoamylase) are essential in starch liquefaction and saccharification processes required in food (baking and brewing) and bioethanol industries (Sindhu et al., 2018; Duan et al., 2019) to make superior quality products such as prebiotics, bread, high-maltose syrup, supplementary diet constituents, and medically significant (e.g., digestive aids, antidiabetic, antihypertensive agents) oligosaccharides derived via transglycosylation reaction (Taniguchi & Honnda, 2009; Ahmad et al., 2019; Mondal et al., 2022). Furthermore, the amylolytic activity of *Bacillus aerius* may contribute to its probiotic and antifungal effect (Niyomukiza et al., 2023) as demonstrated in amylases obtained from plants (Vijayan et al., 2012; Sudam & Sushma, 2018; Shafi et al., 2017; Kirisanth et al., 2020). Amylases from *Bacillus aerius* could also find application in the detergent industry for removing starchy food stains. Deductively, this specie of bacteria could be widely applied in rigorous enzymatic processes and could be better harnessed with genetic modifications (Gopinath et al., 2017).

Arthrobacter sp. A2, on the other hand, grew optimally and exhibited maximum amylase activity at 37 °C defining its appropriateness for moderate temperature applications. Previous studies had investigated amylases with low to moderate temperature activity from farm/plantation soil isolates; *Arthrobacter psychrolactophilus* (Smith & Zahnley, 2005) and *Arthrobacter kerguelensis* (Munaganti et al., 2015). Reports on *Arthrobacter* sp. in the food industry appear limited to its ability to produce β -galactosidase for lactose fermentation in milk processing. However, low-temperature active amylase from *Arthrobacter* sp. is useful in detergent formulation, and Solihin et al. (2021) reported a strain that produces synergistic antibiofilm efficiency with proteases. In their study, Han et al. (2021) revealed that the genus *Arthrobacter* consists of bacteria commonly found in cold environments. They clarified that this physiological function is mediated by specific genes in these bacteria, particularly those that encode proteins like glycogen hydrolysing-glycoside hydrolases (GH) 1, GH 13(α -amylase), CBM48; consequently, *Arthrobacter* spp. are expected to have a variety of low-temperature applications.

CONCLUSION

Using the short-read 16S rDNA PCR amplification technique, we identified the isolates obtained from soil samples of bakery waste dumpsites. One of the isolates was identified at the genus level as *Arthrobacter* sp., while the other was identified at the species level as *Bacillus aerius* through

phylogenetic analysis and morphology. The *Bacillus aerius* isolate exhibited optimal growth and amylolytic activity at a temperature of 45 °C, whereas the *Arthrobacter* sp. isolate thrived best at an incubation temperature of 37 °C; this suggests they have different application areas in starch-based processes; apparently, the *Bacillus aerius* isolate will be more suitable for high-temperature applications and the *Arthrobacter* sp. isolate for low-temperature applications. In future research, we plan to investigate these isolates' full-length 16S rRNA gene sequence and optimize the culture and amylase production conditions using various carbon sources, including bakery waste.

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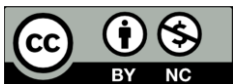
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