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Original Article

Isolation and characterization of amylase-producing *Bacillus* sp. ACoL20 isolated from the gut of flying fish, *Cheilopogon intermedius*

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Abstract: Bacterial enzymes associated with the gut of fish are known to aid in the digestion and nutrition of the host. In the present study, amylase-producing bacteria were isolated, identified, and characterized from the gut of flying fish (*Cheilopogon intermedius*). Isolation was performed on starch agar (SA) plates, and the isolated bacterial strains were qualitatively and quantitatively screened for amylase production. Among the isolates, strain ACoL20 exhibited the highest amylase activity, reaching 15.21 $U.mg^{-1}$ protein. Morphological, biochemical, physiological, and molecular analysis revealed that the isolate belongs to the genus *Bacillus* (86% homologous to *B. paramycoides)*. Further qualitative assessment, through the measurement of the zone of hydrolysis, indicated that optimum amylase production occurred at 36 h at 40°C, with a wide range of pH tolerance (pH 6-9) when cultured in starch agar supplemented with 1% NaCl. These findings highlight the amylolytic potential of *Bacillus* sp. ACoL20 and its possible application in enhancing fish nutrition and other biotechnological processes.

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Introduction

The gastrointestinal (GI) tract of fish is a rich source of diverse and novel enzymes that aid in the breakdown of complex dietary components, including proteins, carbohydrates, and lipids (Nayak, 2010; Ray, 2012; Chakrabarti et al., 2015). It also harbors a diverse group of microorganisms that play vital roles in the host's digestion (Simora et al., 2015; Armada and Simora, 2016), nutrition (Ghosh et al., 2010), and overall health (Nayak, 2010; Ray et al., 2012). Among these enzymes, amylases, which catalyze the hydrolysis of starch and other polysaccharides, are of particular industrial and biotechnological importance (SenGupta et al., 2012; van der Maarel et al., 2002). In global enzyme production, amylase-producing microorganisms contribute approximately 25-35% (van der Maarel et al., 2002, Balkan and Ertan, 2007; Ousaasi et al., 2021), highlighting their immense commercial and industrial potential. These enzymes can be obtained from bacteria, fungi, and yeast, as well as from genetically modified microbes, and are found in various sources, including animals, plants, and microorganisms (Kathiresan and Manivannan, 2006; Murakami et al., 2008).

The flying fish (*Cheilopogon intermedius*) is a small pelagic fish species found near the surface of all tropical and temperate marine waters (Simora et al., 2020). Despite its abundance, this species is considered a low-value fish commodity, often unexploited and underutilized. This is primarily because it has a high bone-to-meat ratio, resulting in less edible flesh; a tendency to form histamine (Simora et al., 2015); its consumption is mostly limited to the flesh; and the lack of adequate processing technologies to enhance its shelf life or market value. However, its diverse diet, typically

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composed of phytoplankton and zooplankton (McGrouther, 2021), suggests a rich and dynamic gut microbiota capable of producing a variety of extracellular enzymes. Exploring the microbial diversity within its digestive system not only provides insight into its nutritional physiology but also uncovers potential sources of industrially valuable enzymes from low-value marine fish commodities.

Among bacterial genera, members of *Bacillus* are particularly noteworthy for their adaptability to diverse environmental conditions, which influences their metabolic activity and enzyme production (Ajayi and Fagade, 2006; Oyeleke and Oduwole, 2009). These gram-positive, spore-forming bacteria are widely used in biotechnology for their ability to secrete large quantities of extracellular enzymes, including amylases (Oyeleke and Oduwole, 2009). A large quantity (20-25 g/l) of extracellular enzymes has been produced and secreted by various *Bacillus* strains, which have placed them among the most significant industrial enzyme producers (Promita et al., 2013). Major industries utilizing these enzymes include detergents (37%), textiles (12%), starch processing (11%), baking (8%), and animal feed (6%), which together account for approximately 75% of all industrial enzyme applications (Schallmey et al., 2004; Das et al., 2011). Further characterization of culture conditions, such as incubation time, temperature, pH, and NaCl concentration, is also crucial to achieving cost-effective enzyme production (Iram et al., 2021; Sharif et al., 2023).

Investigating amylase-producing bacteria from the gut of flying fish (*C. intermedius*) offers benefits as it enhances understanding of digestive processes in marine species and provides potential microbial resources for enzyme-based applications. Furthermore, using these bacteria from low-value yet abundant fish commodities offers a sustainable approach to producing biotechnological products from underexploited marine resources. Therefore, this study aimed to isolate, characterize, and identify amylase-producing bacteria from the gut of flying fish (*C. intermedius*), through biochemical, molecular, and phylogenetic analyses, with the ultimate goal of exploring their potential as a source of amylase for biotechnological applications.

Materials and Methods

Sample collection and bacterial isolation: Wild market-size flying fish (*C. intermedius)* were collected from San Jose de Buenavista, Antique, Philippines (10.7483°N, 121.9508°E) in June 2024. The fish samples were packed in polystyrene boxes with ice and transported to the Microbiology Laboratory of the Western Institute of Technology for processing. Upon arrival, the fish were starved for 24 h and subsequently dissected aseptically following the method of Simora et al. (2015) with minor modifications. Prior to dissection, the ventral surface of each specimen was swabbed with 1% iodine solution to sanitize the surface. The entire gut was removed aseptically, and adherent (autochthonous) bacteria associated with the gut wall were isolated by rinsing the gut tissue thoroughly with sterile 0.9% saline solution (10:1 w/v). Gut homogenates were diluted in sterile 0.9% saline, and appropriate dilutions (up to 10-7) were poured onto starch agar (SA, HiMedia) plates at pH 7.0. Total heterotrophic bacterial count was also determined using tryptone soy agar (TSA, HiMedia) plates at pH 7.0. All plates were incubated at 30°C for 24 h. Distinct bacterial colonies growing on SA plates were subcultured onto SA slants and maintained at 4°C for subsequent analysis.

Screening of extracellular amylase production: All bacterial isolates obtained from SA plates were screened for extracellular amylase production using the starch hydrolysis method described by Simora et al. (2015). The 24-hour-old bacterial cultures were spot-inoculated onto SA medium and incubated at 30°C for 24 h. Following incubation, plates were flooded with an iodine solution to visualize starch hydrolysis. The formation of a clear halo zone surrounding the bacterial colony indicated amylolytic activity. The degree of hydrolysis was assessed based on the diameter of the clear zone and scores as follows: 0-3 mm = +, 4-6 mm = ++, moderate= +++, high, >10 mm = ++++ following the criteria of

Askarian et al. (2012) and Simora et al. (2015) with modifications.

Quantitative amylase activity: Bacterial isolates exhibiting the largest starch-hydrolysis zones were selected and subcultured in starch-containing broth (agar-free selective medium), as previously described, to produce crude extracellular amylase. Quantitative estimation of amylase activity was performed according to Bernfeld et al. (1955), in which 1 unit of amylase activity was defined as the amount of enzyme that liberated 1 mg of maltose in 3 min under the specified assay conditions.

For the enzyme assay, 1 ml of 1% (w/v) starch solution prepared in 20 mM sodium phosphate buffer (pH 7.0) was pre-equilibrated at 55°C in a water bath. Subsequently, 1 ml of the enzyme solution (diluted in ultra-pure water) was added, and the mixture was incubated for exactly 3 min. The reaction was terminated by adding 1 ml of DNS reagent, followed by heating in a boiling water bath for 15 min. The tubes were then cooled rapidly in an ice bath, and 9 ml of ultrapure water was added to each tube. The absorbance of the resulting solution was measured at 540 nm against a blank containing all reagents except the enzyme.

A standard maltose solution was prepared by adding 0.5, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 ml of a 0.2 (w/v) maltose standard to a tube. Ultra-pure water was added to bring the final volume to 2 mL for all standards. To each tube, 1 ml of DNS reagent was added, followed by boiling in a water bath for exactly 15 min. The solution was cooled to room temperature and diluted with 9 ml ultrapure water. Absorbance was read at 540 nm against a reagent blank. The concentration of maltose released in the test samples was determined by linear regression analysis of the standard curve. Protein concentration in the enzyme sample was measured by Lowry's assay (Lowry et al., 1952). All assays were performed in triplicate. Amylase activity was calculated using the following equations:

Enzyme Activity (Units/mL enzyme) = mg of maltose released x Dilution factor

Specific Activity (Units/mg protein) = (Units/mL enzyme) / (mg/ml protein)

Morphological, physiological, and biochemical characterization of the amylase-producing bacterial isolate: The most promising amylase-producing bacterial isolate, as determined by qualitative and quantitative enzyme assays, was subjected to morphological, physiological, and biochemical characterization. Morphological and biochemical characterization was performed according to standardized protocols recommended by the American Society for Microbiology (ASM) (Smith and Hussey, 2005; Breakwell et al., 2007; MacWilliams, 2009a, b; McDevitt, 2009; Shields and Cathcart, 2011; Dela Cruz and Torres, 2012; Lal and Cheeptham, 2012; Reiner, 2012). Macroscopic colony characteristics—such as color, form, elevation, margin, opacity, endospore formation, and texture—were documented. Microscopic examination involved Gram staining and observation of cell morphology under light microscopy at 400× and 1000× magnification. Motility was determined by inoculating sulfide-indole-motility (SIM) medium with a stab and observing growth radiating from the stab line after incubation for one week. The enzymatic profile of the isolate was assessed for the production of catalase (3% H_2O_2), amylase (2% starch agar), protease (1% skim milk agar), gelatinase (12% nutrient gelatin), and urease (urea broth). Biochemical characterization included indole and hydrogen sulfide production (SIM medium), methyl red and Voges–Proskauer (MR–VP broth), citrate utilization (Simmons citrate agar slant), and glucose and acid fermentation (triple sugar iron agar slant) tests. The results were interpreted in accordance with the standard ASM protocols. Antibiotic sensitivity tests for 3 antibiotics were performed using the Himedia HiComb™ disc according to the method of Ruangpan and Tendencia (2004).

Molecular identification of the amylase-producing bacterial isolate: Genomic DNA of the selected amylase-producing bacterial isolate (ACoL20) was extracted from a 24 h old culture grown on a 5 ml tryptone soy broth using PureLink™ Genomic DNA Kit (Invitrogen, USA) following the instructions of the

Table 1. Total heterotrophic and amylolytic count of flying fish (*Cheilopogon intermedius*).

Total bacterial count LVC.g^{-1} intestines	
Heterotrophic count	8.16±0.03 [a]
Amylolytic count	8.09±0.10 [b]

manufacturer. Extracted gDNA was analyzed using NanoDrop spectrophotometry to ensure sample quality. The 16S rRNA gene was amplified using the universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1429R (5′-GGT TAC CTT GTT ACG ACT T-3′). Polymerase chain reaction (PCR) was carried out in a reaction mixture containing 0.2 µM of each primer, 2 µL of template DNA, and 1x Taq Master Mix (Vivantis, Malaysia), which included Taq DNA polymerase, reaction buffer, dNTPs, and MgCl2. Amplification was performed in a thermocycler (S1000TM Bio-Rad, USA) under the following conditions: denaturation at 95°C for 3 min; 32 cycles of denaturation at 95°C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension at 72°C for 3 min. The resulting PCR amplicons were purified and sent for Sanger sequencing (Macrogen, Korea).

The resulting sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to identify closely related sequences in the NCBI GenBank database (blast.ncbi.nlm.nih.gov). Type strains of closely related species that are validly published under the ICNP and listed with correct nomenclatural status in the LPSN database (https://lpsn.dsmz.de/genus/bacillus) (Parte et al., 2020) were downloaded from NCBI (www.ncbi.nlm.nih.gov) and included in the phylogenetic analyses. Multiple sequence alignment was performed using MUSCLE (Edgar, 2004; Cuccuru et al., 2014) and trimmed with TrimAl (Gap threshold 0.8, Similarity 0.5, Consistency 0.4) (Capella et al., 2009) through the Galaxy platform (https://usegalaxy.eu/). Phylogenetic relationships were inferred using the Maximum Likelihood method and the Tamura-Nei model. The robustness of the resulting tree topology was assessed using bootstrap analysis with 1000 replicates. Evolutionary analyses were conducted in MEGA12 utilizing up to 4 parallel computing threads (Kumar et al., 2024). A partial sequence of the isolate was deposited in the NCBI GenBank database to obtain an accession number

Effects of physicochemical factors on amylase production: The effects of various physicochemical factors on amylase production by the bacterial isolate were evaluated under different growth conditions. The parameters tested included incubation time (12, 24, 36, 48, 60, and 72 h), temperature (20, 30, 40, 50, and 60ºC), NaCl concentration (1, 2, 3, 4, and 5%), and pH (5, 6, 7, 8, 9, and 10). For each parameter, bacterial cultures were incubated in starch-containing broth adjusted to the specified conditions. Amylase production was qualitatively assessed by measuring the diameter of the hydrolysis zone around the colony on starch agar, expressed in millimeters (mm).

Statistical analysis: All data expressed as means were analyzed using One-way analysis of variance (ANOVA) followed by Tukey's test using Statistical Package for the Social Sciences (SPSS) version 20.0. $P<0.05$ were considered statistically significant.

Results

Analysis of the total heterotrophic and amylolytic counts of adherent bacteria isolated from the gut of *C. intermedius* revealed significantly ($P<0.05$) dense bacterial populations in both categories (Table 1). The mean heterotrophic bacterial count was 8.16±0.03 log viable count. g^{-1} (LVC·g^{-1}), while the amylolytic bacterial count was slightly lower at 8.09±0.1 LVC·g^{-1}. Despite the small numerical difference, statistical analysis indicated a significant difference ($P<0.05$) between the two groups, suggesting that not all culturable gut bacteria possess amylolytic activity.

The intensity of extracellular amylase production, as assessed by the qualitative enzyme assay (Table 2), revealed amylolytic activity in 20 distinct bacterial colonies isolated from the gut of *C. intermedius*. Based on starch degradation incorporated with 1% NaCl, 9 out of 20 colonies (45%) showed positive amylase activity scores, indicating their ability to

Table 2. Qualitative enzyme activity of adherent amylolytic bacteria isolated from the gut using starch agar incorporated with 1% NaCl.

Bacterial Isolate	Amylase Activity Scores
ACoL1	+
ACoL2	+
ACoL3	+++
ACoL4	+
ACoL5	++
ACoL6	++
ACoL7	+
ACoL8	++
ACoL9	+
ACoL10	++
ACoL11	+
ACoL12	++
ACoL13	+
ACoL14	+
ACoL15	+
ACoL16	+
ACoL17	++
ACoL18	++
ACoL19	+
ACoL20	++++

Values are expressed as mean ± SD (n=3). 0-3 mm = +, 4-6 mm = ++, moderate= +++, high, >10 mm = ++++

hydrolyze starch. Among these, isolates ACoL3, ACoL5, ACoL6, ACoL8, ACoL10, ACoL11, ACoL12, ACoL17, ACoL18, and ACoL20 showed measurable starch-degrading ability, with ACoL20 producing the highest activity score (++++) followed by ACoL3 (+++) and ACoL5 (++), indicative of moderate amylolytic activity.

The top three amylase-producing bacterial isolates identified by qualitative screening, ACoL3, ACoL5, and ACoL20, were subsequently subjected to a quantitative enzyme assay to determine their specific amylase activities. As shown in Table 3, isolate ACoL20 significantly exhibited the highest specific amylase activity (15.21 U.mg protein^{-1}), followed by ACoL3 (6.55 U.mg protein^{-1}) and ACoL5 (4.20 U.mg protein^{-1}) (P<0.05). Based on qualitative and quantitative results, isolate ACoL20 was identified as the most potent amylase producer and was thus selected for further taxonomic identification and characterization.

The morphological, physiological, and biochemical characteristics of isolate ACoL20 are summarized in Table 4. Morphologically, the isolate appeared as a creamy-white, circular, raised colony with a smooth texture, and large, rod-shaped cells that stained Gram-positive and were capable of producing endospores. Physiological characterization showed that the isolate grew well at temperatures between 20 and 40°C and tolerated salt concentrations up to 5%. Biochemical tests revealed that ACoL20 could hydrolyze substrates such as starch, but was unable to liquefy casein or gelatin. It also utilized glucose and arabinose as carbon sources but not mannitol, lactose, or sucrose. In antibiotic sensitivity tests, the isolate was sensitive to novobiocin but resistant to ampicillin.

While morphological and biochemical analyses provided preliminary evidence that isolate ACoL20 belongs to the genus *Bacillus*, these traditional methods alone are often insufficient for accurate species-level identification because of overlapping phenotypic traits among closely related taxa. To confirm its identification, 16S rRNA gene sequencing was performed, and the resulting sequence was compared with reference strains available in the NCBI GenBank database. Molecular identification based on 16S rRNA gene sequencing confirmed that isolate ACoL20 belongs to the genus *Bacillus*. BLAST analysis of the obtained sequence revealed its closest similarity (86%) with *B. paramycoides* (accession no. KJ812444) and *B. pseudomycoides* (AF013121), indicating a close phylogenetic relationship with these taxa but potentially representing a distinct or previously uncharacterized strain within the *Bacillus* group. The phylogenetic analysis using the neighbor-joining method (Fig. 1) further supported this relationship, showing that ACoL20 formed a distinct cluster with *B. paramycoides*, supported by a bootstrap value of 77%, confirming its affiliation with the genus *Bacillus*. The sequence was deposited in GenBank with accession number PX490823.

Physical factors such as incubation time, temperature, pH, and NaCl concentration were evaluated to determine their effects on the amylase activity of *Bacillus* sp. ACoL2 (Figs. 2a–2d). The

Table 3. Quantitative enzyme activity of adherent amylolytic bacteria isolated from gut starch broth incorporated with 1% NaCl.

Bacterial Isolate	Specific amylase activity (U.mg^{-1})
ACoL3	6.55 ± 0.25 [b]
ACoL5	4.20 ± 0.20 [a]
ACoL20	15.21 ± 1.50 [c]

Values are expressed as mean±SD (n=3). Values with the same superscripts are not significantly different (*P*>0.05); U = µg of tyrosine liberated/mg protein/ml of culture filtrate.

Table 4. Morphological, physiological, and biochemical characteristics of ACoL20 isolated from the gut of flying fish (*Cheilopogon intermedius*).

Test	Study	Results
Morphology	Microscopic study	Gram positive, rods, endospores
	Colony morphology (48 h incubation on nutrient broth, 37ºC)	Circular, creamy white color, entire/lobed margin, raised elevation, smooth texture, opaque
Physiological test	Growth on nutrient broth Motility in SIM agar	Growth on temperature range 20ºC-40ºC, Salt tolerance range 0%-5%
	Other physiological tests	+ve: Catalase -ve: Voges-Proskauer test, oxidase, citrate
Biochemical test	Substrate hydrolysis	+ve: Starch -ve: Gelatin, casein
	Acids from sugar	+ve: Glucose, arabinose -ve: Mannitol, lactose, mannose, inositol, sucrose
	Antibiotic sensitivity assay	Sensitive: Novobiocin (30 ug) Resistant: Ampicillin (10 ug), 0129 (10 ug), 0129 (150 ug)

+ve: positive; -ve: negative

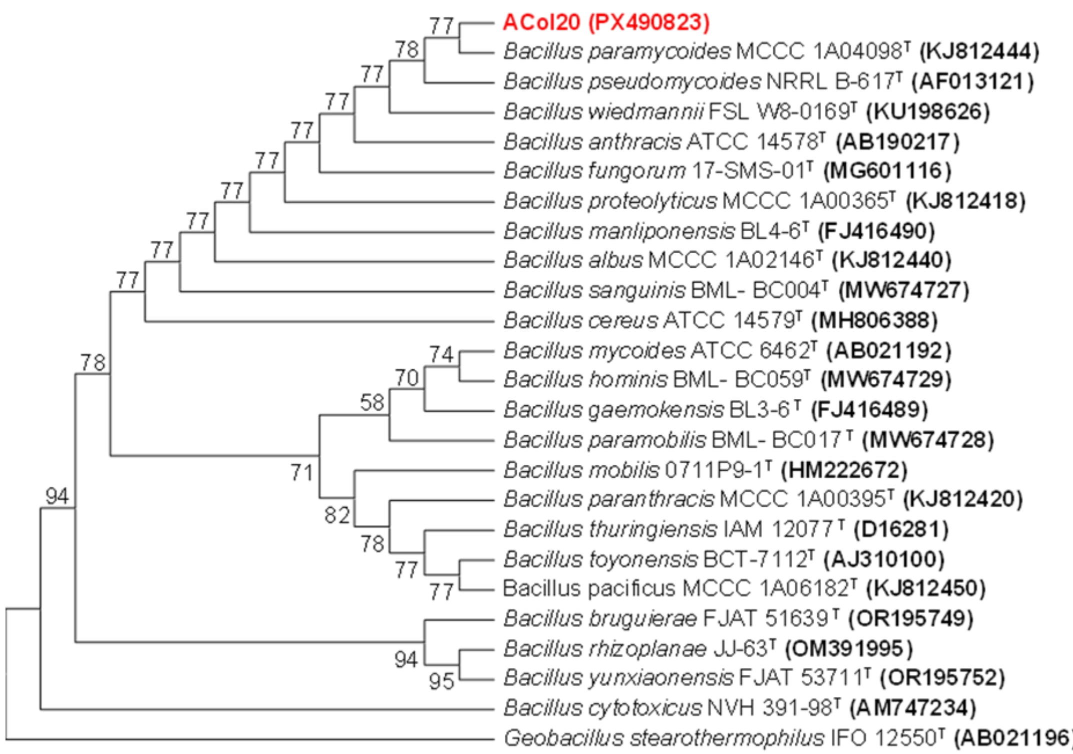

Figure 1. Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequences, constructed under the Tamura–Nei model, showing the topology only and the taxonomic position of isolate ACoL20 among the closely related type strains of the genus Bacillus. Bootstrap values of >50 % are shown as percentages of 1000 replications (GenBank accession numbers are given in parentheses. There were a total of 1395 positions in the final dataset.

results revealed that the tested conditions highly influenced enzyme activity. Amylase activity significantly increased (*P*<0.05) with incubation time, reaching a maximum at 36 h after which a decline was observed. Enzyme activity rose steadily with increasing temperature, attaining a significant

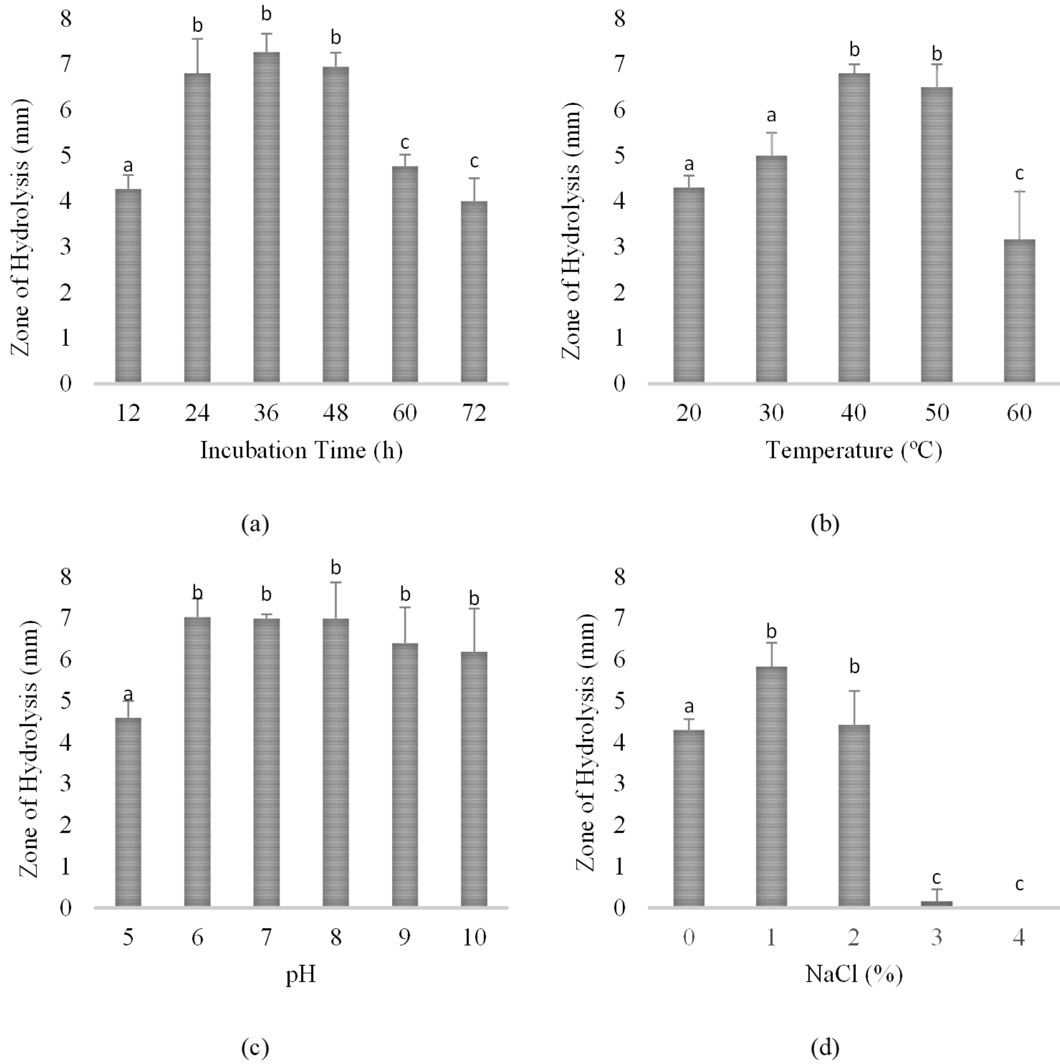

Figure 2. Effect of (a) incubation time, (b) temperature, (c) NaCl concentration, and (d) pH with reference to amylase production from isolate ACoL20. Values are expressed as mean±SD (n=3).

maximum ($P<0.05$) at 40°C, and then gradually decreased at higher temperatures. The isolate exhibited optimal amylase production at pH 6, with substantial activity maintained from pH 7 to 9 ($P>0.05$), indicating broad pH tolerance. Likewise, for NaCl concentration, the highest activity was observed at 1% NaCl, followed by 2%, whereas enzyme production was significantly ($P<0.05$) reduced at 3% and completely inhibited at 4%.

Discussions

Bacteria are abundant in aquatic environments, making their ingestion by fish an inevitable component of the diet (Ray et al., 2012). The total heterotrophic count in the current study reflects the overall microbial carrying capacity of the GI tract, encompassing both enzyme-producing and non–enzyme-producing microorganisms contributing to host digestion (Simora et al., 2015; Armada and Simora, 2016), nutrient cycling (Nayak et al., 2010), and competitive exclusion of pathogens (Ray et al., 2010). In the present study, the high bacterial load observed in the gut of *C. intermedius* indicates an abundant, metabolically active microbiota, typical of marine fish species exposed to microbially rich environments (Egerton et al., 2018). This finding aligns with previous studies demonstrating the abundance of heterotrophic bacteria in fish GI systems, where they contribute to host metabolic activities (Nayak, 2010; Ray et al., 2012; Simora et al., 2015). Furthermore, earlier reports by Simora et al. (2015) on rabbitfish (*Siganus guttatus*) suggested that the gut provides a favorable environment for microbial colonization.

The presence of high amylolytic count in the gut of *C. intermedius* may also reflect a dietary inclination of this species towards plant-derived materials (vander Maarel et al., 2002), since amylase is primarily involved in carbohydrate degradation (vander Maarel et a., 2002; Gupta et al., 2003). The presence of amylolytic bacteria supports the enzymatic breakdown of dietary carbohydrates, consistent with findings that carbohydrate-hydrolyzing bacteria such as *Bacillus* spp. and *Pseudomonas* spp. common in the intestines of marine fish (Luang-In et al., 2019; Kim et al., 2021). The same feeding behavior and associations have been observed in herbivorous and omnivorous fish species such as several freshwater riverine species (SenGupta et al., 2012), rohu (Kar and Ghosh, 2008), and various carps (Bairagi et al., 2002; Ray et al., 2010), reinforcing the widespread occurrence of amylase-producing microorganisms across diverse fish species.

The presence of a clear halo around bacterial growth on SA plates indicates the secretion of extracellular amylase (Hankin and Anagnostakis, 1975; Gupta et al., 2003). Based on the present study, the positive results for these isolates suggest that a significant portion of the gut microbiota degrades carbohydrates, further supporting the fish's digestive capacity for starch-rich diets. Similar findings have been reported in other herbivorous and omnivorous fish species, where gut-associated bacteria contribute to the enzymatic breakdown of dietary starches, thereby enhancing host nutrient utilization (Kar and Ghosh, 2008; Ray et al., 2010; Saha et al., 2006). The results of the current work were further supported by quantitative measurement of amylolytic activity in the top three amylase producers. This amylolytic activity falls within the typical range (0.05-20 U.mg protein^{-1}) reported for *Bacillus* species producing extracellular amylase under similar conditions (Sodhi et al., 2005; Oyeleke et al., 2010; Abdel-Azeem et al., 2021). The different amylase activity among the isolates could be attributed to environmental factors such as salt concentration, pH, and substrate availability (Murakami et al., 2008; Sharif et al., 2023).

The higher amylase activity of ACoL20 compared with other isolates (ACoL3 and ACoL5) implies that ACoL20 may be well adapted to the marine gut environment of *C. intermedius*, where moderate salinity and polysaccharide-rich diets prevail. A study by Supriya et al. (2020) reported a specific amylase activity of 2.16 U.mg protein^{-1} using the DNS assay from *Bacillus cereus* isolated from the gut of bullhead catfish (*Ameiurus melas*), whereas higher activities were isolated from *Bacillus* sp. DA 5.2.3 resulting in an amylase activity of 47.3 U.mg protein^{-1} isolated from shrimp (Jamilah et al., 2009). The observed amylase activity among the isolates suggests that ACoL20 is a moderate-to-strong amylase producer, and the presence of these amylase-producing bacteria underscores their potential importance in the digestive ecology of *C. intermedius*.

Based on morphological, physiological, and biochemical characterization, ACoL20 exhibited features typical of the genus *Bacillus*. The ability to form endospores further supports its classifications, as this characteristic is a key taxonomic trait of the genus (Logan and De Vos, 2015). The ability to hydrolyze starch substrate also confirms the isolate's potential role in carbohydrate digestion, aligning with reports that *Bacillus* species commonly produce extracellular hydrolytic enzymes, including amylases, to assist in nutrient breakdown (Das et al., 2014; Sarkar et al., 2020). The catalase-positive reaction indicates the microorganism's ability to detoxify hydrogen peroxide, which is a common trait of aerobic *Bacillus* strains (Ray et al., 2012). Antibiotic sensitivity testing revealed that isolate ACoL20 was sensitive to novobiocin (30 μg) but resistant to ampicillin (10 μg) and compound 0129 (10 and 150 μg). This resistance to antibiotic patterns is consistent with previous findings that certain *Bacillus* species, particularly environmental and marine isolates, exhibit inherent or acquired resistance to antibiotics such as ampicillin due to the presence of β-lactamase enzymes (Sumi et al., 2015). In terms of carbohydrate utilization, the isolate ACoL20 fermented glucose and arabinose but not mannitol, lactose, or sucrose, suggesting a selective carbohydrate metabolic profile. According to Logan and De Vos (2015), such variability in sugar fermentation among *Bacillus* species is useful as a taxonomic marker for species-level differentiation. ACoL20 also demonstrated growth across a broad temperature range (20-40°C) and tolerated NaCl concentrations up to 5%. These characteristics indicate that the isolate is mesophilic and moderately halotolerant, enabling it to survive and function in the marine gut environment of *C. intermedius*. Similar growth characteristics and adaptability have been reported for *B. subtilis* and *B. licheniformis* strains isolated from the intestines of marine fish (Sugita et al., 1997; Hoshino et al., 2018).

Overall, the morphological, biochemical, and molecular analyses support the classification of isolate ACoL20 as a member of the genus *Bacillus*. It is well known that the genus *Bacillus* produces a wide variety of extracellular enzymes, of which amylases are of particular industrial importance (Swain et al., 2006). The predominance of *Bacillus* among the isolates is consistent with previous studies reporting this genus as a major contributor to enzyme production in fish intestines (Ray et al., 2012; Das et al., 2014; Simora et al., 2015). *Bacillus* species are also well recognized for secreting extracellular amylases that facilitate the degradation of complex carbohydrates (Sarkar et al., 2020). This is consistent with several studies by Askarian et al. (2012), Das et al. (2014), and Mondal et al. (2010). The occurrence of other *Bacillus* sp., such as *B. cereus*, within the gut of Atlantic salmon (*Salmo salar* L.) with promising enzyme activity was also reported previously by Askarian et al. (2012).

The phylogenetic analysis based on 16S rRNA gene sequences revealed that isolate ACoL20 clustered closely with *B. paramycoides*, confirming its taxonomic affiliation within the *B. cereus* group. The placement of ACoL20 alongside other amylolytic *Bacillus* species highlights its evolutionary linkage to known starch-degrading bacteria, suggesting a conserved enzymatic function within this lineage. Overall, the morphological, biochemical, and physiological and molecular traits of isolate ACoL20 strongly suggest its affiliation with the genus *Bacillus*, and its moderate to high amylolytic activity implies a significant role in the digestive ecology of *C. intermedius*, particularly in carbohydrate degradation.

It is known that amylase production in *Bacillus* sp. ACoL20 can be influenced by environmental factors, including temperature, pH, and salinity (Sivaramakrishnan et al., 2006; Souza and Magalhaes, 2010). In the present study, the enzyme showed maximum activity at 40°C, indicating its thermophilic nature, consistent with findings in *Bacillus* spp. reported by Esakkiraj et al. (2009) and Aygan et al. (2008). The decrease in amylase activity above 40°C may be due to thermal denaturation or reduced metabolic efficiency (Ray et al., 2007). Amylase production was optimal at pH 6 and remained active under alkaline conditions (pH 7-9), suggesting the enzyme's stability across a broad pH range. Lower pH values (pH 5) led to reduced activity, likely due to enzyme inactivation or disruption of hydrogen ion balance (Castro et al., 1993). Similar pH optima were observed in *B. amyloliquefaciens* strains producing α-amylase at pH 6-7 (El-Tayeb et al., 2007; Nusrat and Rahman, 2007). Salt (NaCl) concentration also affected enzyme production, with optimal activity at 1% NaCl and inhibition at higher levels (3-4%). This indicates moderate halotolerance, a common trait among marine *Bacillus* isolates (Nair et al., 2008). Low salt concentrations may enhance enzyme stability or secretion, while excessive NaCl can induce osmotic stress, reducing enzymatic yield (Reddy et al., 2003; Shafiei et al., 2010). This temperature profile supports the classification of ACoL20 as a mesophile suited to the natural conditions of the host's digestive tract (Madigan et al., 2018; Prescott et al., 2020).

Conclusion

The results of the present study indicate a distinct microbial source of extracellular amylases, distinct from endogenous sources, in the digestive tract of *C. intermedius*. The most promising strain, ACoL20, belonging to the genus *Bacillus,* exhibited maximal amylase production at 36 h of incubation, an optimum pH of 6, and a 1% NaCl concentration. The enzyme also exhibited tolerance across a wide pH range and moderate salinity, as well as moderate thermal stability, suggesting its adaptability to diverse environmental conditions. These findings indicate that *Bacillus* sp. ACoL20 is a promising candidate for industrial and biotechnological applications, particularly in processes that require thermostable, moderately halotolerant amylases. Moreover, this study highlights the value of using low-value yet abundant species such as *C. intermedius* as sustainable reservoirs of functional microorganisms for enzyme production and other marine biotechnological innovations, warranting further optimization and purification studies.

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