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Characterization of amylase-producing *Streptomyces* sp. NAA-28 isolated from mangrove sediment, Red Sea, Egypt

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ABSTRACT
Hydrolytic enzymes produced by actinomycetes are of outstanding quality and have important industrial uses. *Streptomyces* sp. NAA-28, an amylase- producing bacterium, was isolated from mangrove sediments and was chosen for comprehensive characterization and optimization of amylase production, based on its higher amylolytic activity as determined by the formation of a clear zone using starch agar medium. The strain was identified based on the biochemical and morphological characteristics and the sequence of the 16s rDNA gene. The sequence was deposited in GenBank under the accession number MZ496703. The optimum NaCl concentration for amylase production was 6%. The strain produced the highest amount of amylase after 5 days of incubation at 35°C in the culture medium adjusted to pH 8 and supplemented with peptone and starch as carbon and nitrogen sources, respectively. Further studies are required to purify and characterize the secreted enzyme to determine its potential for industrial applications.

INTRODUCTION

Enzymes are essential components of living cells, as they catalyse the metabolic pathways to produce biomolecules that maintain the living system **(Shoda *et al.* 2016)**. The catalytic potential of enzymes from living organisms is exploited for the development of varying industrial bioprocesses. Enzymatic bioprocesses are preferred over chemical methods in the chemical industry because they work under mild reaction conditions (e.g., atmospheric conditions, pH, temperature), have high stereo-selectivity, yielding stereo- and regio-chemically defined reaction products at high acceleration, have a long half-life, do not require substrate functional group protection, and work on unnatural substrates **(Johnson, 2013)**. Hydrolases (EC 3 in enzyme commission classification) are widespread among microorganisms, especially bacteria **(Quax, 2006)** and fungi **(Beltagy *et al.* 2022)**. These enzymes are important for both the natural ecosystem, where they contribute to the geochemical processes through the breakdown and modification of complex macromolecules, and the industrial sector, as they are important components in the pharmaceutical, agricultural, and food industries **(Singh *et al.* 2016)**.
Microbial enzymes are important for many industries, including pharmaceuticals, pulp and paper, detergents and textiles, leather, animal feed and personal care, chemicals, food and beverages, biofuels, among others **(Adrio and Demain, 2014)**. In industry and medicine,

microbial enzymes are favored over plant and animal enzymes due to the low cost and less time of production, stability at extreme conditions, less toxicity and ease of control of their co-compounds, and ease of production and optimization **(Anbu, 2013; Anbu and Hur, 2014; Gopinath *et al.* 2015)**. Actinomycetes, which are a significant component of the microbial populations in plant tissues, soil, and marine environments, produce a variety of enzymes which are used in various industries, including, proteases, cellulases, lipases, amylases, xylanases, chitinases, pectinases, and cutinases, **(Mukhtar *et al.* 2017)**. Among microbial enzymes, marine-derived enzymes are active under harsh conditions (high-salt concentration, wide range of temperature and pH, and high incidence of light and pressure) and constitute unique biocatalysts **(Birolli *et al.* 2019)**.

Amylases are enzymes that catalyse the hydrolysis of starch into sugars. These enzymes have a wide application in industry and constitute about 25% of the enzyme market **(Rao *et al.* 1998)**. In the starch hydrolysis industry, amylases have almost replaced chemical methods, with microbial amylases having the broadest spectrum of uses in food, fermentation, textile, and paper industries **(Gurung *et al.* 2013)**. The amylases from *Streptomyces* spp. are used in different industries and constitute about 25% of the enzyme market **(Mukhtar *et al.* 2017)**. The amount of amylase produced by microbes is largely affected by the cultivation parameters including, cultivation method, pH, nutrient requirements, incubation time and cultivation temperature **(Al-Dhabi *et al.* 2020).** In this study, the isolation and characterization of *Streptomyces* sp. NAA-28, an amylase-producing strain were described, which was isolated from the mangrove rhizosphere 103 kilometers north of Marsa Alam on the Egyptian Red Sea coast. The effect of the physical and nutritional growth parameters on the bioprocess of amylase production was described.

MATERIALS AND METHODS

Sample collection

Using a sterile scooper, four Sediment samples were collected from the rhizosphere of *Avicinia marina* plants at Sharm el-Bahari mangrove area located 103 km north Marsa Alam and 33 km south El- Quseir between latitude 25°52′2.91″N and longitude 34°24′ 47.71″E **(Figure 1)**. The collected samples were stored in sterile polyethylene bags at 4 °C until return to the laboratory (**Kafilzadeh and Dehdari 2015**)

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Isolation of actinomycetes

For isolation of actinomycetes, ten grams of each sediment sample were homogenized in 90 mL of sterilized seawater and serially diluted up to 10^{-5}. The serial dilutions of all samples were plated on starch casein nitrate agar prepared using 50% seawater and supplemented with nalidixic acid (25 mg/L) and cycloheximide (75 mg/L). The plates were incubated for 14 days at 30°C. Actinomycete colonies were selected and purified by streaking on the same medium.

Screening for amylase production

To screen the actinomycete isolates for amylase production, all isolates were cultured on starch nitrate agar medium and incubated for 7 days at 30 °C. Amylase-positive isolates were identified by the appearance of a clear zone on a blue background after the plates had been inundated with iodine solution **(Elmansy *et al.* 2018)**. The diameters of the clear zones and colonies were measured in mm **(Pranay *et al.* 2019)**. The amylolytic index was calculated as the ratio between the diameter of clear zone and the diameter of the colony **(Islam *et al.* 2016)**. The isolate with the greatest amylase activity was chosen for further characterization studies.

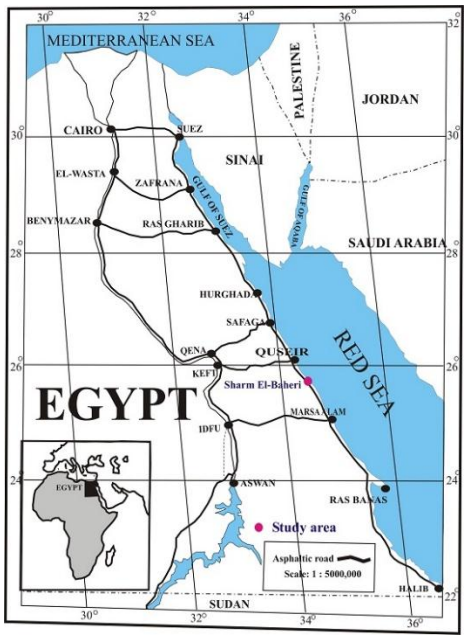

Figure 1. A map of Egypt showing the location of Sharm el-Bahari mangrove area **(Madkour *et al.* 2014).**

Characterization of the most potent amylase producer

The morphological and physiological characteristics of the most potent strain were extensively studied. The strain was grown under different culture conditions, including different NaCl concentrations, different pH values, and different temperatures. Production of diffusible pigment, the color of aerial and substrate mycelia, and the utilization of carbon sources were analyzed as described by **Shrilling and Gottlieb (1966)**. The morphology of the spore chain and the spore surface texture were observed by scanning electron microscopy **(Kumar *et al.* (2011)**. Nitrate reduction, gelatin liquefaction, and the production of extracellular hydrolytic enzymes (amylase, cellulase, protease, and lipase) were determined as described by **Williams *et al.* (1983)**. The sensitivity of the strain to 10 antibiotics was evaluated using the disc diffusion method **(Kumar *et al.* 2014)**.

Molecular identification

PCR amplification of the 16s rDNA gene was carried out using two primers, Strep F; 5`-ACGTGTGCAGCCCAAGACA-3` and Strep R; 5`-ACAA GCCCTGGAAACGGGGT-3`, according to **Edwards *et al.* (1989)**. The PCR product was purified using quick PCR purification reagents (Qiagen, USA) and sequenced. The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed to evaluate the similarity. Multiple sequence alignment and phylogenetic tree construction were performed using the Mega-X software, version 10.1.7 **(Khan *et al.* 2011; Hall, 2013)**.

Optimization of amylase production

The factors influencing the production of amylase by strain AAN-28 grown in starch nitrate agar were determined using one factor at a time method **(El-Batal *et al.* 2016)**. The optimized parameters were as follows: NaCl concentration (0-10%), pH (6-11), temperature (20-40 °C), carbon source (glucose, starch, lactose, mannitol, sucrose, fructose, and glycerol), nitrogen source (yeast extract, ammonium sulphate, potassium nitrate, urea, and peptone), and incubation time (1-10 days).

Enzyme assay

Amylase activity was determined using the culture supernatant obtained after centrifugation. The reaction mixture (0.5 mL) consisting of 100 μL of cell-free supernatant and 0.4 mL of starch solution in phosphate buffer (pH 7) was incubated at 30 °C for 30 min. The reaction was stopped by placing the mixture in boiling water for 5 minutes **(Mohapatra *et al.* 2003)**. The amount of the reducing sugar liberated was estimated using the dinitrosalysilic acid method **(Miller 1959)**. The quantity of amylase that liberates 1 μg/mL of glucose per minute was considered as one unit of amylase activity.

RESULTS

Isolation of actinomycetes

In the present study, the isolation of actinomycetes from mangrove sediment yielded eleven actinomycetes isolates (NAA-20- NAA-30). Using starch casein nitrate agar medium prepared using 50% seawater, all of the isolates were tested for the production of amylase **(Table 1)**. Due to the highest starch hydrolysis activity of NAA-28 isolate in comparison with the other isolates, it was chosen for further characterization and optimization of amylase production **(Figure 2).**

Morphological and biochemical characterization of strain NAA-28

The NAA-28 strain is aerobic, Gram-positive, and filamentous, and has a spiral spore chain and a warty spore surface. The strain produced catalase, and urease, hydrolyzed starch, carboxymethyl cellulose, gelatin, and Tween 80. It reduced nitrate and utilized glucose, fructose, maltose, starch, glycerol, and starch as a sole carbon source but failed to utilize lactose, xylose, or arabinose. The strain grew at a range of 0-10% of NaCl at a temperature range of 20-50 °C and a pH range of 6–9. Among the antibiotics tested, the strain resisted Nalidixic acid 30 mcg, Ampicillin 10 mcg, Amoxicillin 5 mcg, Tetracycline 30 mcg, Ofloxacin 5 mcg, and Levofloxacin 5 mcg **(Tables 2)**.

Table 1: Amylase screening results of actinomycete isolates

Strain code	Average amylase activity (mm)	Average colony diameter (mm)	Amylolytic index
NAA-20	25	11	2.27
NAA-21	23	12	1.92
NAA-22	18	10	1.80
NAA-22	20	15	1.33
NAA-23	14	8	1.75
NAA-24	17	9	1.89
NAA-25	21	8	2.63
NAA-26	18	11	1.64
NAA-27	22	10	2.20
NAA-28	32	11	2.9
NAA-29	25	13	1.92
NAA-30	26	13	2.00

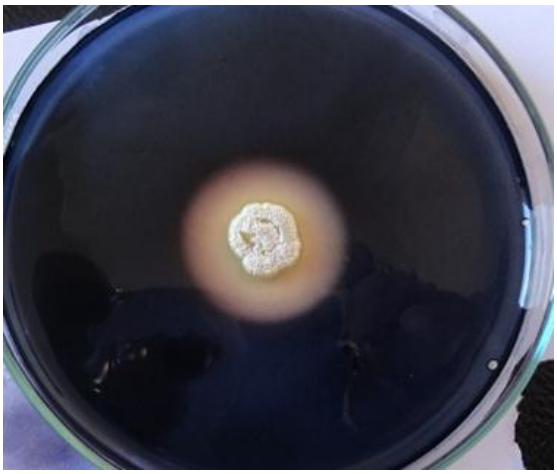

Figure 2. Amylase activity of NAA-28 isolate after 7days of incubation. Amylase activity is shown by the development of the clear zone around the bacterial colony grown in starch casein nitrate agar plate after treatment with Lugol's iodine reagent.

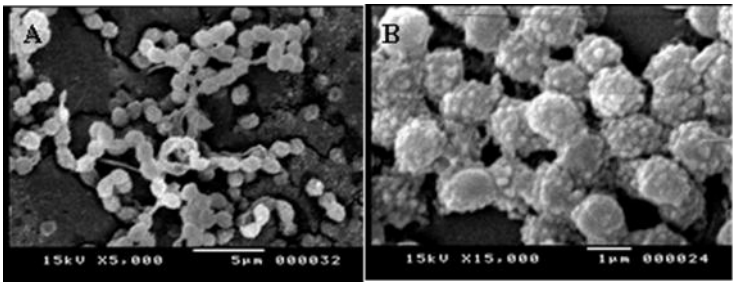

Figure 3: Scanning electron micrograph of NAA-28 strain showing a spiral spore chain (A) and a warty spore surface (B).

Molecular phylogeny of strain NAA-28

The 16s rDNA was amplified using PCR to determine the taxonomic position of the strain NAA-28. The amplified product was sequenced and compared to the sequences in the NCBI nucleotide database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). The BLAST search revealed that the strain belongs to the genus *Streptomyces* and showed high similarity to many species of the genus: *Streptomyces* sp. strain JJ28 (GenBank accession no. KX352773, 99.2% similarity), *Streptomyces* sp. AML554 (GenBank accession no. AJ486874, 99.2% similarity), *Streptomyces* sp. AML250 (GenBank accession no. HQ873928, 99.2% similarity), and *Streptomyces mangrovicola* strain PH23 (GenBank accession no. MT669288, 99.2% similarity). The sequence was deposited in the GenBank as *Streptomyces* sp. strain NAA-28 with an accession number of MZ496703 **(Figure 4)**.

Optimization of amylase production

Effect of NaCl concentration

The impact of sodium chloride concentration (0–9%) on the ability of strain NAA-28 to produce amylase was studied. The strain produced the highest level of amylase in the medium containing 6% NaCl. However, higher NaCl concentrations decreased the production of amylase **(Figure 5)**.

Table 2: Morphological, biochemical and physiological characteristics of NAA-28 strain

Characteristic	Result	Characteristic	Result
Morphological characteristics		Starch	+
Gram	+	Glycerol	+
Spore chain	Spiral	Sucrose	+
Spore surface	Warty	**Resistance to:**	
Diffusible pigment	Brown	Nalidixic acid 30 mcg	+
Substrate mycellium	yellow	Erythromycin 15 mcg	-
Aerial mycelium	Green	Ampicillin 10 mcg	+
Physiological and biochemical properties		Amoxicillin 5 mcg	+
Production of:		Tetracyclin 30 mcg	+
Cellulase	+	Amikacin 30 mcg	-
Amylase	+	Gentamycin 10 mcg	-
Lipase	+	Ofloxacin 5 mcg	+
Gelatinase	+	Streptomycin 10 mcg	-
Urease	+	Levofloxacin 5 mcg	+
Catalase	+	**Growth at different pH**	
Nitrate reduction	+	5	-
Utilization of carbon sources		6-9	+
Glucose	+	**Growth at different NaCl (w/v, %)**	+
Fructose	+	0-10	+
Maltose	+	11	-
Lactose	-	**Growth at different temperature**	
Arabinose	-	15°C	-
Xylose	-	20- 50 °C	+

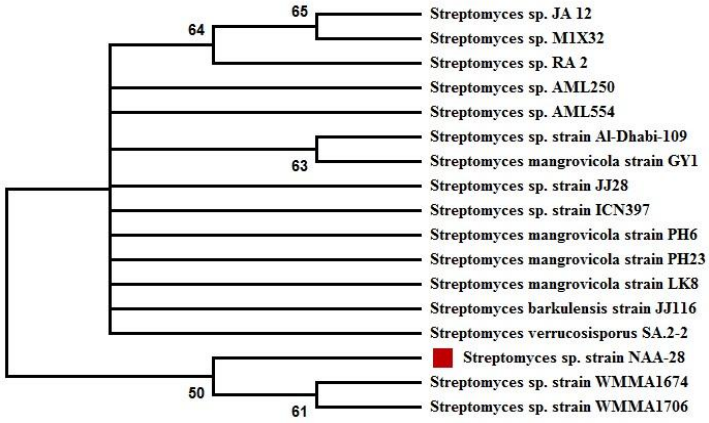

Figure 4: Neighbor-joining phylogenetic tree based on 16S rDNA sequences of NAA-28 strain and related species. Numbers at nodes are bootstrap values (> 50%) based on 1000 resamplings.

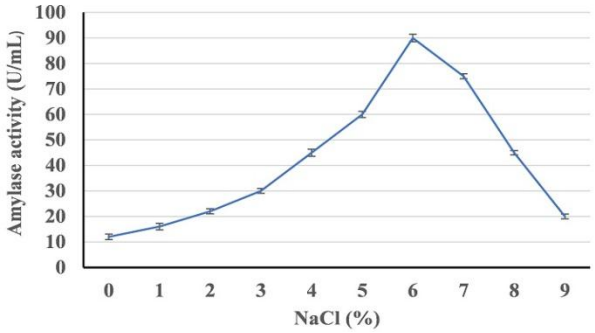

Figure 5: Effect of sodium chloride concentration on the production of amylase by strain NAA-28 after incubation for 5 days at 35 °C under shaking conditions (150 rpm). Values are means of three replicates and standard deviations are presented as error bars (n=3).

Effect of pH, temperature, and incubation period

Figure 6 shows the effect of the incubation temperature (20–50 °C) on the production of amylase by the NAA-28 strain. The optimum temperature for the production of amylase was found to be 35 °C (80 U/mL). With increasing growth temperature, there was a drastic decrease in enzyme production, with the enzyme yield dropping to 8 (U/mL) at 50 °C. Concerning the effect of pH, strain NAA-28 was able to produce amylase over a wide pH range (6-10) with maximum enzyme production at pH 8 **(Figure 7)**. Amylase production was found to be maximal after incubating the culture for 5 days (65 U/mL± 1.2) and decreased with increasing the incubation time **(Figure 8).**

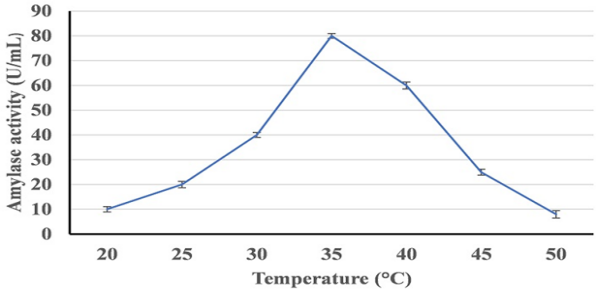

Figure 6: Effect of temperature on the production of amylase by strain NAA-28 after incubation for 5 days under shaking conditions (160 rpm). Values are means of three replicates, and standard deviations are presented as error bars (n=3).

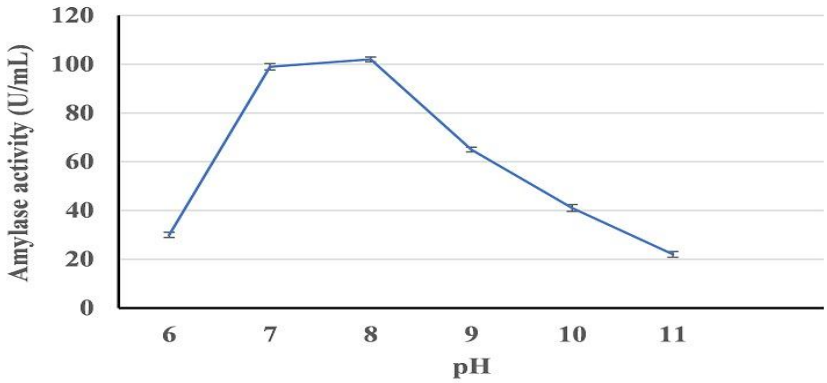

Figure 7: Effect of pH on the production of amylase by strain NAA-28 after incubation for 5 days at 35 °C under shaking conditions (160 rpm). Values are means of three replicates.

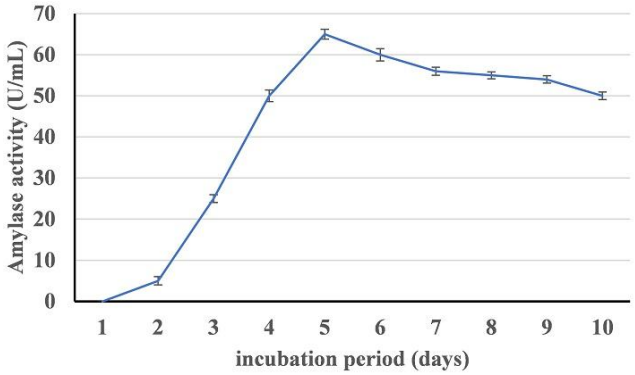

Figure 8: Effect of incubation period on the production of amylase by strain NAA-28 after at 35 °C under shaking conditions (160 rpm). Values are means of three replicates, and standard deviations are presented as error bars (n=3).

Effect of carbon and nitrogen sources

The effect of carbon and nitrogen sources on the production of amylase by strain NAA-28 was studied. As shown in Figure 8, amylase production was found to be maximal in the presence of starch in the culture medium as a carbon source (90 ± 1.3 U/ml). With regard to the effect of nitrogen sources, the culture medium supplemented with peptone supported the highest level (88 ± 1.1 U/ml) of amylase production **(Figure 9)**.

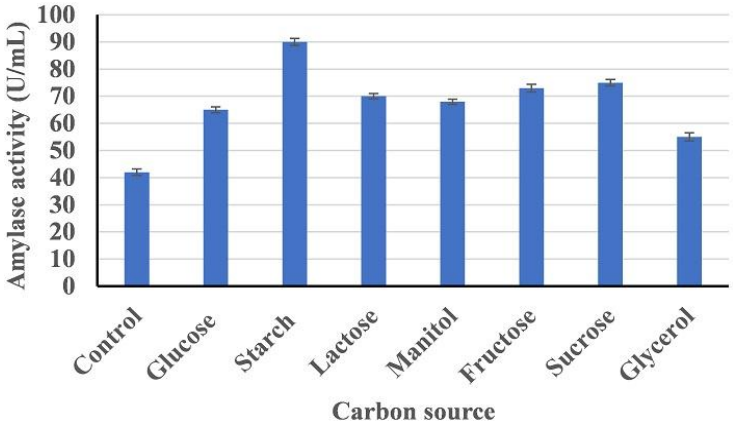

Figure 9: Effect of carbon sources on the production of amylase by strain NAA-28 after incubation for 5 days at 35 °C under shaking conditions (160 rpm). Values are means of three replicates, and standard deviations are presented as error bars (n=3).

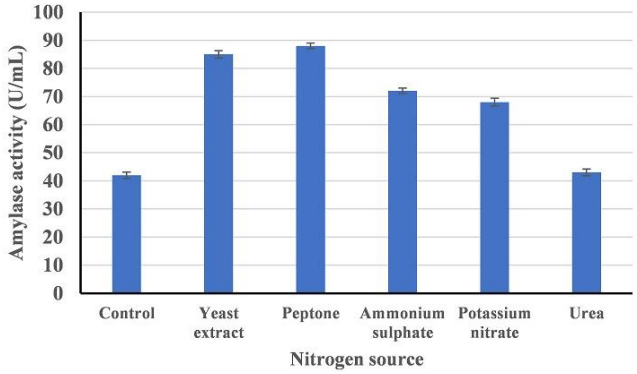

Figure 10: Effect of nitrogen sources on the production of amylase by strain NAA-28 after incubation for 5 days at 35 °C under shaking conditions (160 rpm). Values are means of three replicates, and standard deviations are presented as error bars (n=3).

DISCUSSION

A total of eleven actinomycete were isolated from mangrove sediment and one isolate was selected, coded as strain NAA-28, on the basis of its better potential to produce amylase, for further characterization and optimization of the production of amylase. The strain was identified as a species of the genus *Streptomyces* and designated as *Streptomyces* sp. NAA-28 with the GenBank accession number MZ496703. The results showed that the strain NAA-28 produces the highest yield of amylase in the medium supplemented with 6% NaCl, starch and peptone as a carbon and nitrogen sources, respectively and incubated for 6 days at 35 °C.

The mangroves are extraordinary type of forests distributed along the intertidal zone around the world **(Giri *et al.* 2011**). Despite the harsh environmental conditions in mangroves (high temperature, high salinity, and high sedimentation), theses environments are highly productive and important ecosystem. Mangrove ecosystem sustains biodiversity by providing a nursery habitat for a broad range of flora and fauna **(Carter *et al.* 2015)**. Mangrove ecosystem harbor a highly diverse microbial community including various species of bacteria and fungi. In mangrove sediment, bacteria are the most important decomposers of organic matter and, therefore, increase the amount of nutrients which stimulate the growth of various organisms and promote species diversity **(Ghizelini *et al.* 2012)**. The bacterial population of mangrove sediment play an important ecological role in phosphate solubilization, nitrogen fixation, enzyme production, sulphate reduction, anoxygenic photosynthesis, and methanogenesis **(Sahoo and Dhal 2009; Ghizelini *et al.* 2012)**. *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Acidobacteria*, *Cyanobacteria*, *Nitrospirae*, *Planctomycetes*, and *Fusobacteria* are the dominant bacterial phyla in mangrove ecosystem **(Wang *et al.* 2012; Thompson *et al.* 2013; Basak *et al.* 2016; Priya *et al.* 2018)**.

Actinomycetes from mangrove ecosystems have been acquiring a great interest from researchers around the world due to their inconceivable capabilities to produce bioactive compounds and enzymes **(Law *et al.* 2019)**. Among actinomycete genera, *Streptomyces* is widely distributed in mangrove environments **(Lee *et al.* 2014; Ser *et al.* 2018)**, and has frequently been identified as the dominating genus in actinomycete populations recovered from marine sediment **(Kafilzadeh and Dehdari 2015; Rosmine and Varghese 2016; Retnowati *et al.* 2017)**. According to

Bergey's manual of systematic bacteriology **(Bergey *et al.* 1989)**, most of the actinomycetes isolated during the current investigation belonged to the genus *Streptomyces*. According to our findings, the majority of the isolates showed starch hydrolysis activity. Several studies were carried out in relation to the production of useful enzymes by microorganisms. **Kafilzadeh and Dehdari (2015)** studied the potential of actinomycetes isolated from mangrove sediments in Iran and found that the majority of the isolates produced amylase and belonged to the genus *Streptomyces*. **Mamangkey *et al.* (2021)** investigated the potential of the culturable bacteria isolated from mangrove ecosystems in North Sumatra and found multiple potentials of the isolated bacteria to produce extracellular hydrolytic enzymes such as amylase, cellulase, chitinase, phosphatase, protease, and urease. **Ramesh and Mathivanan (2009)** reported the ability of aquatic actinomycetes isolated from the Bengal Gulf to produce industrial enzymes such as caseinase, lipase, amylase, gelatinase, and cellulase. **Peela *et al.* (2005)** isolated industrial enzyme-producing streptomycetes from the Andaman Islands coast. Also, **Selvin *et al.* (2009)** reported the production of industrial enzymes (cellulase, lipase, and amylase) by the three types of actinomycetes isolated from the southwest coast of India.

The physical factors and the composition of the culturing medium influence the growth and the production of extracellular hydrolytic enzymes by actinomycetes. Therefore, optimization of physical factors and medium components is urgently needed to increase the productivity of bacterial enzymes **(Al-Dhabi *et al.* 2020)**. The optimum conditions for the production of amylase by strain NAA-28 were investigated. The obtained data revealed that the strain produced the greatest amount of amylase when the culture medium was supplemented with starch and peptone as carbon and nitrogen sources, respectively. **van Hille *et al.* (2009)** reported that supplementing the culture medium with peptone, yeast extract, and tryptone enhanced the production of amylase However, **Al-Dhabi *et al.* (2020)** found that beef extract was the best nitrogen source for the production of amylase by *Streptomyces* sp. Al-Dhabi-46. Concerning the effect of the different carbon sources on amylase production, strain NAA-28 secreted the highest level of amylase when starch was supplemented to the culture medium. These findings are in agreement with **Al-Dhabi *et al.* (2020)**. Although starch is the best carbon source for the majority of bacterial species to produce amylase, other sugars such as glucose, fructose, maltose, and lactose produce the highest yield of amylase amylase **(Suman and Ramesh, 2010; Ashwini and Gaurav, 2011)**. After five days of incubation at 35 °C, strain NAA-28 displayed the maximum amylase activity when cultured in a culture medium with a pH of 8. The physiological behaviour of the various species affects the ideal pH and temperature for amylase production. According to **Al-Dhabi *et al.* (2020)**, *Streptomyces* sp. Al-Dhabi-46 produced the most amylase after being incubated for five days at 40 °C and pH 8. Since strain NAA-28 is halotolerant in nature, sodium chloride concentration affected the production of amylase and the maximum yield was attained at 6% sodium chloride.

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