Investigating Starch Degradation Efficiency and Key Attributes of Soil Bacteria from Makandura (NWP) Area

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1 Introduction

Starch is a polysaccharide composed of many glucose monomers linked together by glycosidic bonds. Amylase, an enzyme crucial in carbohydrate metabolism, catalyses the breakdown of starch into sugars. The two forms of amylase are alpha and beta amylase. Amylases are among the most useful enzymes in the industrial enzyme sector, representing nearly 25% of the market [1]. They are widely used in industries such as food, detergents, textiles, and paper for starch hydrolysis during various processes.

Although amylases can be derived from plants, animals, humans and microorganisms, those originated from microorganisms dominate industrial use [2]. This is mainly because of their exceptional stability with higher genetic diversity, high enzymatic activity, and ease of manipulation necessary for the efficient production of enzymes with the desired characteristics [3]. Hence, many microorganisms have been identified and chosen as sources of amylase production.

Soil harbours a high concentration of amylolytic bacteria, resulting in a plentiful production of amylase enzymes. This abundance can fulfil the demand for amylase enzymes in various industries [4]. Despite the extensive study of amylase activity in soil bacteria, there is a need to explore specific microbial populations in different geographical regions. In Sri Lanka, where amylase is mostly imported, identifying effective amylase producing local strains for industrial uses is important.

Therefore, this study aimed at isolating, and characterizing starch degrading bacterial strains from microbial populations in the soil of the Makandura area to uncover novel amylase-producing bacterial strains that could offer unique properties and potential industrial applications.

2 Methodology

2.1 Sample Collection

Soil samples were collected from three different locations in the Makandura area, which is in the North-Western province of Sri Lanka, in November 2022. The topsoil was taken by sterile spatula from 2-3 cm depth and transferred into a sterile zip-lock bag and immediately taken to the laboratory. The samples were stored at 4 °C for further steps.

2.2 Sample Preparation and Isolation of Bacteria

This was done according to the method described by Dida [5]. Ten grams of each soil sample were mixed with 90 ml of sterile peptone water, homogenized on an orbital shaker, and serially diluted up to 10^{-5}. Aliquots (0.1 ml) from each dilution were plated on nutrient agar plates and incubated at 37°C for 48 hours.

2.3 Screening and Purification of Starch Degrading Bacteria

Primary Screening of Potential Amylase Producing Bacterial Isolates

Bacterial colonies with distinct morphologies were picked from different dilution plates and spot inoculated onto replica plates with nutrient agar and 2% soluble starch agar plates to screen for amylolytic properties. These plates were incubated at 37°C for 24 hours. After the incubation, starch agar plates were treated with Lugol's iodine solution (5 mM I_2 and 5 mM KI) [6]. Isolates that formed clear zones, indicating starch hydrolysis, were selected from the master plate for secondary screening.

Secondary Screening and Obtaining Pure Colonies of Potential Amylase Producing Bacteria

The isolates from primary screening were streaked onto nutrient agar and incubated at 37°C for 24 hours. Single colonies were then inoculated on starch agar plates along with a negative control (*Escherichia coli* JM109). After incubating at 37 ^{0}C for 24 hours, the iodine test was performed for each isolate, and the plates were observed for clear zones. Colony and clear zone diameters were measured. The isolates were further purified by repeated streaking and incubation to obtain pure colonies.

2.4 Determination of Starch Degrading Index

The iodine test was replicated three (03) times for each isolate. The starch degrading index (SDI) was calculated by the ratio of the diameter of the clear zone to the mean diameter of the colony. This was used to describe the starch degrading ability of the bacterial isolates [5].

2.5 Characterization of Bacterial Isolates

Morphological Characterization

The bacterial isolates were streaked on nutrient agar plates and incubated at 37 ^{0}C for 24 hours. Colony characteristics describing size, shape, texture, opacity, and pigmentation were observed macroscopically. For the microscopic characterization, Gram staining and motility tests were performed following Dash and Payyappilli [7], and Ningthoujam and Shovarani [8].

Biochemical Characterization

Biochemical tests were conducted for the further characterization of bacterial isolates. The catalase and KOH tests followed protocols by Dash and Payyappilli [7] and Silaban et al. [4], respectively. Indole production, citrate utilization, methyl red and Voges - Proskauer tests were performed as per Masi et al. [9]. The triple sugar iron test (TSI) was done according to the protocol of Stager et al. [10]. For the identification of isolated bacterial strains, Bergey's Manual of Systematic Bacteriology [11] and Bergey's Manual of Determinative Bacteriology [12] were consulted.

2.6 Statistical Analysis

Analysis of Variance and mean separation using Tukey's test were performed to analyse the data using Minitab Statistical Software version 20.3.

3 Results and Discussion

3.1 Isolation and Primary Screening

Several colonies were obtained from soil samples after performing the serial dilution. Based on the colony morphology, 47 colonies were selected from the 10^{-2} dilution spread plate. After inoculation and performing the iodine test, 12 isolates displayed a zone of clearance around their colonies. The formation of clear zones around the colonies indicated the absence of starch in the clear zone, suggesting the presence of potential amylase producing bacteria.

3.2 Secondary Screening

These isolates were picked from the replica plate, streaked, and an iodine test was performed. Based on the starch degrading index, seven potential isolates were selected for further study. They were subjected to subsequent streaking on nutrient agar seven times and iodine testing on starch plates, to obtain pure isolated colonies (**Fig. 1**).

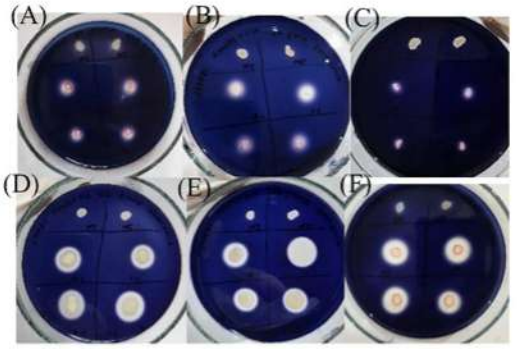

Fig. 1. Zone of hydrolysis of starch by the potential amylase producing bacteria. (A) Isolate 02; (B) Isolate 03; (C) Isolate 09; (D) Isolate 20; (E) Isolate 21; (F) Isolate 37

3.3 Determination of Starch Degrading Index

The mean diameters of the clear zones and the starch degrading index of isolates after secondary screening are given in Table 1. The starch degrading index was statistically significantly different ($p<0.05$) between the colonies. According to the table, the isolates with the best efficiency based on their starch degrading index were 02, 09 and 37 having SDIs of 3.52 ± 0.29, 3.90 ± 0.46 and 3.77 ± 0.79 respectively.

Table 1. Starch degrading index (SDI) of isolated colonies

Isolate Number	Mean diameter of colony (cm)	Mean Diameter of clear zone (cm)	SDI
02	0.29 ± 0.02^{cd}	1.03 ± 0.07^{b}	3.52 ± 0.29^{a}
03	0.48 ± 0.05^{c}	1.09 ± 0.10^{b}	2.30 ± 0.13^{b}
09	0.17 ± 0.03^{d}	0.64 ± 0.05^{c}	3.90 ± 0.46^{a}
20	0.82 ± 0.03^{b}	1.55 ± 0.14^{a}	1.90 ± 0.19^{b}
21	0.97 ± 0.22^{ab}	1.48 ± 0.15^{a}	1.56 ± 0.23^{b}
24	1.05 ± 0.07^{a}	1.58 ± 0.04^{a}	1.51 ± 0.13^{b}
37	0.43 ± 0.07^{c}	1.56 ± 0.12^{a}	3.77 ± 0.79^{a}

3.4 Characterization of the Bacterial Isolates

The colony characteristics, microscopic observations and biochemical characteristics of the bacterial isolates are summarized in Table 2.

When considering the colony characteristics, creamy orange and creamy pink pigmentation were observed in isolates 03 and 02 respectively.

Out of the seven bacterial isolates, four (57.1%) were gram positive and three (42.8%) were gram negative. All the gram-positive bacterial isolates (02, 03, 20 and 21) were rod shaped. Out of the gram-negative bacterial isolates, 09 and 24 were rod shaped and isolate 37 was spherical (**Fig. 2**). Gram negativity was further confirmed by the 3% KOH test in which isolates 09, 24 and 37 showed positive results. Isolates 02, 03, 20, 21, 24 and 37 were motile while isolate, 09 was non-motile.

According to the biochemical tests, only isolates 20, 21 and 24 were positive for citrate utilization. The MRVP (Methyl Red - Voges-Proskauer) test showed that only isolates 09 and 24 were positive for the methyl red test, while isolates 03 and 24 were positive for the Voges-Proskauer test. All the isolates showed positive results for starch hydrolysis. Isolates 09, 20, 21, 24 and 37 were catalase positive, while isolate 02 was negative. Isolate 03 showed variable results for the catalase test. Only isolate 24 showed a positive result for the indole test. For the triple sugar iron test, isolate 24 showed an alkaline slant and an acid butt, while isolate 37 showed an alkaline slant and an alkaline butt. There was no colour change in the reaction for the other isolates.

Following the comparison of the colony morphology, microscopic observations, and results of the biochemical tests of isolates 20 and 21 with the studies of Bergey et al. [11], it was identified that the bacterial isolates 20 and 21 belonged to the genus *Bacillus*. The current finding is consistent with the study of Dida [5]. Also based on Holt et al. [12], isolate 24 was classified as a bacterial species that belonged to the genus *Enterobacter*. This is in agreement with the previous study by Luang-In et al. [13]

Table 2. Colony, microscopical and biochemical characteristics of bacterial isolates

Characteristic	Isolate						
	02	03	09	20	21	24	37
Colony							
Size	medium	medium	small	large	large	large	medium
Shape	round	round	round	round	round	round	round
Texture	smooth	smooth	smooth	rough	rough	smooth	smooth
Opacity	translucent	translucent	transparent	opaque	opaque	opaque	translucent
Pigmentation	creamy pink	creamy orange	white	whitish	whitish	whitish	creamy white
Microscopic							
Shape	rod	rod	rod	long rod	long rod	rod	sphere
Arrangement	single, chains	single, chains	single	single, chain, clusters	single, chain, clusters	single, clusters	single, double, clusters
Gram reaction	positive	positive	negative	positive	positive	negative	negative
Motility	motile	motile	non-motile	motile	motile	motile	motile
Biochemical							
KOH test	-	-	+	-	-	+	+
Catalase test	-	d	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	+	-
Methyl Red test	-	-	+	-	-	+	-
Voges-Proskauer	-	+	-	-	-	+	-
Citrate utilization	-	-	-	+	+	+	-
Triple sugar iron test							
Slant	w	w	w	w	w	alkaline	alkaline
Butt	w	w	w	w	w	acid	alkaline
Gas	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-

+, >85% positive; d, variable (16–84% positive); −, 0–15% positive; w, weak reaction.

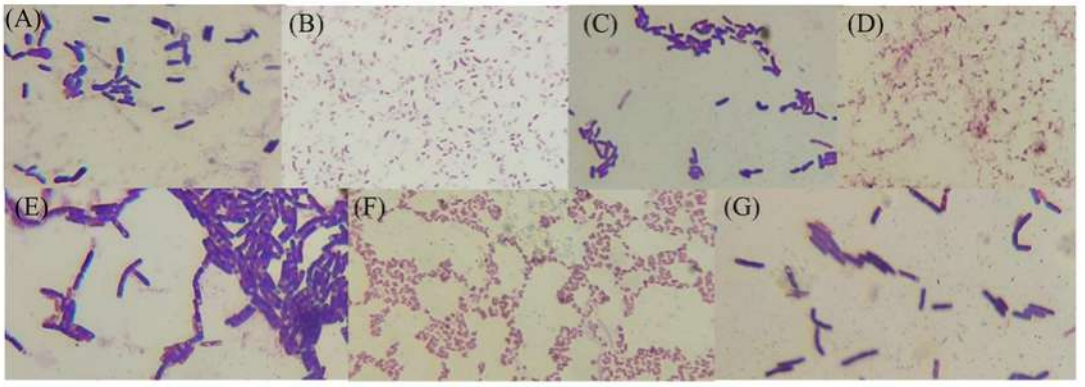

Fig. 2. Microscopic view of the selected amylase producing bacterial isolates after gram staining (A). Isolate 02; (B). Isolate 09; (C). Isolate 03; (D). Isolate 37; (E). Isolate 20; (F). Isolate 24; (G). Isolate 21

Conclusion

The soil was inhabited by a multitude of microorganisms, from which seven potential amylase producing bacterial isolates were isolated, purified, determined the starch degrading index, and characterised. The study found that among them, four isolates (2, 3, 20, and 21) were gram-positive, while isolates 9, 24, and 37 were gram-negative. According to the starch degrading index, the gram-negative isolates 09 and 37 and the gram-positive isolate 02 were considered to have the best starch degrading efficiency. The study successfully identified isolates 20 and 21 as *Bacillus* species and isolate 24 as *Enterobacter* species based on colony morphology, microscopic observations, and biochemical characteristics.

Further studies are suggested to use molecular identification of these strains to verify the present findings, develop recombinant enzymes, and explore the full potential of these soil bacteria for industrial and environmental applications using scaled-up bioreactor conditions.

Keywords: Amylase, Biochemical characterization, Morphological characterization, Soil bacteria, Starch degrading enzymes

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