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Isolation and characterization of starch degrading rhizobacteria from soil of Jimma University Main Campus, Ethiopia

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Starch degrading bacteria are important for different industries such as food, fermentation, textile, and paper. The aim of this study is to isolate and characterize bacteria able to degrade starch from the rhizospheres of various plants at four sites located in Jimma University main campus. Collected soil samples were labeled as kobo (AJUMC), Avocado (BJUMC), Banana (CJUMC), and *Cana indica* (DJUMC) respectively. Soil samples were serially diluted in sterilized peptone water, poured on sterilized starch agar plates, and incubated at 32°C for 48 h. The representative colonies showing different morphology was randomly picked up using the streaking method on nutrient agar. A total of 53 bacterial isolates were obtained from the soils rhizospheres. Microscopic characteristics showed that among the 53 isolates, 38 (72%) were Gram-positive rod shaped bacteria, while 15 (28%) were Gram-negative rod shaped bacteria. Based on the biochemical tests, the results revealed that the 38 isolates belonged to the genera *Bacillus* while the remaining isolates belonged to the genera *Pseudomonas*. All isolates were catalase positive and only 15 isolates (*Pseudomonas*) were KOH positive with negative growth at 80°C, while the 38 (*Bacillus*) isolates have positive growth at 80°C. The highest values of starch degrading index were the Gram positive bacteria isolates. The amylase activity was also carried out with respect to time, temperature and pH of the media. The maximum activity of amylase at different temperatures from 35 to 45°C was recorded at 35°C (0.94 U/ml) within 24 h, while maximum activity at different pH from 5 to 9 was recorded at pH 7 (1 U/ml).

Key words: Rhizobacteria, starch degrading, amylase enzyme, *Bacillus*, *Pseudomonas*.

INTRODUCTION

The diversity of microbes are associated with plant roots (Berendsen, 2012). The narrow zone of soil directly surrounding the root system is referred to as rhizosphere, while the term 'rhizobacteria' implies a group of rhizosphere bacteria competent in colonizing the root

environment (Ahmad and Kibret, 2014). The rhizosphere is soil ecological environment for plant-microbe interactions involving colonization of different microorganisms in associative, symbiotic, naturalistic, or parasitic interactions depending upon plant nutrient

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status in soil, soil environment, and the type of microorganism proliferating in the rhizosphere zone (Kennedy, 2005). When microbes are close to epidermis, plants secrete signal molecules against invasive microbes in the root zone. Consequently, differentiation takes place between pathogenic, symbiotic, or naturalistic adaptation of microbes with the plant (Hayat et al., 2010).

Rhizosphere bacteria, especially species of *Pseudomonas* and *Bacillus* have been identified in the rhizosphere of various leguminous and non-leguminous plants (Halverson and Handelsman, 1991; Parmar, 1999). Different bacterial genera are vital components of soils. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turnover. Ahmad and Kibret (2014) reported that based on the functional activities, rhizobacteria are classified as (i) bio-fertilizers (increasing the availability of nutrients to plant), (ii) phyto-stimulators (iii) Rhizoremediators (degrading organic pollutants), (iv) biopesticides (controlling diseases, by production of different metabolites).

There are various starch degrading microorganisms from different sources (Aiba et al., 1983; Tonkova et al, 1993; Kathiresan and Manivannan, 2006). Rhizosphere bacteria are one source of starch degrading microorganism as it contains mostly starch substrate (Alariya et al., 2013). Among the bacterial species, *Bacillus* spp. *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Pseudomonas* are widely used for enzyme production. Other species like *Bacillus cereus* and *Bacillus subtilis* also used for production of the amylase enzyme. Amylases produced from *B. licheniformis*, *Bacillus stearothermophilus*, and *B. amyloliquefaciens* show promising potential in a number of industrial applications in processes such as food, fermentation, textiles and paper industries. (Konsoula and Liakopoulou-Kyriakides, 2007; Ha et al., 2001). Thus, the objective of the present study is to isolate and characterize the starch degrading bacteria from different soils rhizospheres of Jimma University.

MATERIALS AND METHODS

Description of study area

The study was conducted at Jimma, located 353 km southwest of Addis Ababa, the capital city of Ethiopia. The town's geographical coordinates are 7°41'N latitude, 36°50'E longitude, and an average altitude of 1,780 m above sea level. It lies in the climatic zone locally known as "Woyna Daga" (1,500 - 2,400 m above sea level) which is considered to be ideal for agriculture as well as human settlement. The town is generally characterized by warm climate with a mean annual maximum temperature of 30°C and a mean annual minimum temperature of 14°C. The annual rainfall ranges from 1138 - 1690 mm. The maximum precipitation occurs during the three months period from June through August, with minimum rainfall occurring in December and January. From a climatic point of view, abundant rainfall makes this region one of the best watered of Ethiopian highland areas, conducive for agricultural production (Alemu et al., 2011).

Samples collection

Ten grams of soil were collected from the rhizospheres of various plants at four sites located in Jimma University. The soil sample were put in the sterilized bag and transferred immediately to the laboratory.

Sample preparation and isolation of starch degrading bacteria

Each 10 g of soil samples from different sites was mixed with 90 ml of sterile peptone water in different 250 ml beaker and homogenized in a flask for ten minutes using orbital shaker at 110 rpm. Subsequently, 1 ml of each sample was transferred aseptically into 9 ml of sterile peptone water and mixed thoroughly using vortex. The homogenates was serially diluted up to 10^{-5} ; thereafter, 0.1 ml aliquot of appropriate dilution was spread properly on starch agar plates. The plates were incubated at 32°C for 48 h. The bacterial isolates showing different colonies morphology were picked out and purified. A fourth gram of soil samples was collected from four different sites (10 g from each site) of Jimma University plant rooted by streaking on nutrient agar (Oseni and Ekperigin, 2013).

Identification of bacterial isolates

The bacterial isolates were subjected to identification using morphological characteristics such as Gram reaction and endospore formation according to Silva and Nahas (2002). Biochemical tests including catalase test, KOH test, and the growth at 80°C were also performed (Naik et al., 2008; Williams et al., 1990).

Determination of starch degrading index (SDI)

Ability of the bacterial isolates to degrade starch was described by the starch degrading index (SDI): the ratio of the total diameter of clear zone and colony diameter. On the basis of degrading index, potential colonies with the best efficiency were selected as best starch degrading colonies (Nusrat and Rahman, 2007).

Screening for amylase activity (Starch Iodine Test)

Isolated strains were picked up from each plate containing pure culture and streaked in straight lines in starch agar plates as carbon source. The plates were incubated at 32°C for 48 h. After incubation, the plates were flooded with (Gram's iodine- 250 mg iodine crystals added to 2.5 g potassium iodide solution, and 125 ml of distilled water, to produce a deep blue coloration (Prameela et al., 2016).

Amylase production

Amylase was produced by using complex medium containing starch 1.0%, yeast extract 0.04%, $(\text{NH}_4)_2\text{HPO}_4$ 0.4%, KCl 0.1% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, and semisynthetic medium containing peptone 0.4%, $(\text{NH}_4)_2\text{HPO}_4$ 0.4% and KCl 1.0% (Kim et al., 1995).

Enzyme assay

A suitable volume of isolated broth culture was incubated for 48 h; thereafter, it was centrifuged at 5000 rpm for 20 min. at 4°C. Supernatant was recovered and amylase determined using



Figure 1. Morphology and biochemical tests.

Table 1. Total isolated colonies and their codes from 4 sites of rhizobacteria (RB).

Isolate code	No. of isolates	Plant rhizosphere
AJUMC	17	Kobo
BJUMC	10	Avocado
CJUMC	11	Banana
DJUMC	15	<i>Cana indicia</i>

spectrophotometer. One milliliter of crude enzyme was placed into a test tube and 1 ml of 1% soluble starch in sodium phosphate buffer (pH 7) was added into test tube. The test tubes were covered and incubated at 35°C for 10 min (Miller, 1959). Subsequently, 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 10 min. After cooling at 25°C, final volume was made to 10 ml with distilled water. The absorbance was measured at 540 nm using spectrophotometer.

Determination of optimum pH

One percent of starch was used as a substrate. Four sets of substrate solution were prepared in sodium phosphate buffer with adjusted pH values 5, 6, 7, 8 and 9. One milliliter of each of crude enzyme solution was added into buffer tubes. Mixture was incubated at 35°C for 10 min; thereafter, 2 ml of DNS reagent was added to terminate the reaction and the mixture was incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 10 ml with distilled water and the activity of the enzymes was measured based on the absorbance at 540 nm.

Determination of optimum temperature

One ml of the substrate was distributed into six test tubes and 1 ml of phosphate buffer pH 7 was added in each test tubes. Tubes were marked with different temperature (at 30, 35, 40°C). 1 ml of crude enzyme solution was added in each tube; thereafter, tubes were incubated at specific temperature for 10 min. Reactions were terminated by adding 2 ml DNS reagent and the mixture incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 10 ml with distilled water and the activity of enzymes was determined by taking the absorbance at 540 nm.

Table 2. Morphological features of 53 isolates of rhizobacteria.

Morphology feature	Bacteria isolate	Number
Cell shape	Rod	53
	Chain	41
Colony arrangement	Single bacillus	12
	+	38
Endospore	-	15
	+	38
Gram reaction	-	15

Determination of optimum time

The effect of different time duration such as 24, 48 and 72 h on amylase activity was observed.

Determination of optimum starch concentration

Similarly, to observe the effect of different substrate concentration on amylase activity, the dialyzed aliquot was added to different starch concentrations (0.5, 1.0, 1.5 and 2%) and the activity was observed following the method of Miller (1959).

RESULTS AND DISCUSSION

The morphology and biochemical tests (Figure 1) resulted from four samples of Jimma University main campus plant roots of 53 isolates which were summarized in Tables 1 and 2. In the present study, on the basis of their gram reaction, out of 53 isolates 38(72%) had rod shape Gram positive bacteria while 15(28%) were Gram negative rod shaped bacteria. All the Gram negative were rod shaped, non-spore forming; similarly, Gram positive bacteria were rod shaped and spore former. Biochemically, 53 isolates were catalase

Table 3. Biochemical tests of isolated strains of rhizobacteria (RB).

Isolate code	No. of isolate	KOH test	Catalase test	Growth at 80°C	Starch hydrolysis test	Identification
AJUMC	13	-	+	+	+	<i>Bacillus</i>
AJUMC	4	+	+	-	+	<i>Pseudomonas</i>
BJUMC	7	-	+	+	+	<i>Bacillus</i>
BJUMC	3	+	+	-	+	<i>Pseudomonas</i>
CJUMC	7	-	+	+	+	<i>Bacillus</i>
CJUMC	4	+	+	-	+	<i>Pseudomonas</i>
DJUMC	11	-	+	+	+	<i>Bacillus</i>
DJUMC	4	+	+	-	+	<i>Pseudomonas</i>

positive, only 15 isolates were KOH test positive, whereas all other isolates showed KOH negative (Table 3).

Among the 53 isolates, four were Gram-negative and 13 were Gram-positive isolated from the rhizosphere of kobo (A site) plant. Similarly, seven isolates were Gram-positive and three were Gram-negative bacteria isolated from avocado plant rhizosphere (B site) and also 7 isolates and 4 were Gram-positive and Gram-negative bacteria isolated from banana rhizosphere (C site) respectively. Finally, 11 Gram positive and 4 Gram negative bacteria were isolated from *Cana indica* (D) plant root. As Halverson and Handelsman (1991) and Parmar (1999) reported, by the same token the identification of our isolates based on morphological characteristics and some biochemical tests indicated that the most dominant isolates were Gram positive rod shaped and Gram negative rod shaped of *Bacillus* and *Pseudomonas* which were the main starch degrading rhizobacteria isolated from starch agar plates, respectively. Various species of bacteria, fungi and actinomycetes are most prominent enzyme producers. Among various amylase producers, *Bacillus* species are most prominent. With increase in its application spectrum, the demand is for the enzyme with specificity. This finding agreed with the study of Sasmita and Niranjana (2008) which states that "efficiency of *Bacillus* species to degrade starch shows high efficiency to reduce sugar in the area with factors such as temperature and pH value correlated". Therefore, this result is true and similar to the finding of Sasmita and Niranjana (2008).

The highest of most rhizobacteria showing high value of starch degrading index were mostly Gram positive, spore former bacteria; and enzyme production from microorganism is directly correlated to the time period of incubation (Smits et al., 1996). The highest degradation of starch by rhizobacteria can secrete amylases to the outside of their cells to carry out extra-cellular digestion and facilitate other different organic matters for plants to easily absorb and manufacture their food (Cordeiro et al., 2003). According to Wang et al. (2016), suggesting that the hydrolyzability of the substrate by amylase increased

with increase of starch solubility irrespective of large diameter of the colony formed by Gram negative, non-spore former rhizobacteria. Also, the calculated starch degrading index value formed a range that was very low in relation to Gram positive rhizobacteria of low diameter colony forming; this again indicate the spore forming and rod shape. Gram positive bacteria almost shows characteristics of *Bacillus* species, capable of tolerating different factors, produces amylase enzymes to degrade starch into soluble form and applicable to food, industrial and leather industries (Table 4).

The amylase production of bacteria was tested by starch hydrolysis (Figure 2). On the basis of the area of clearance, characterization, and high amount of amylase activity determined by parameter (pH, temperature, incubation period and concentration of starch) (Table 5) all isolates response showed positive result for pH value, temperature, concentration of starch and incubation period enhanced enzyme activity with the increase in incubation time especially true for *Bacillus* species (Aiyer, 2004).

While amylase activity was recorded at different pH from 5 to 9, it shows maximum amylase activity at pH 7 (1 U/ml) (Figure 3). There is increase in amylase activity at basic pH 9 = 0.88 U/ml and decrease in acidic medium pH 5 = 0.5 U/ml. As Shihui et al. (2016) reported, the enzyme showed good activity over a broad range of temperatures (40 - 80°C) and pH values (3 - 7), indicating that it has potential use in a broad range of food industry applications. Meanwhile, the result obtained correlates this statement and can play a great role in industry applications. The optimum temperature and pH for the enzyme were 35°C and pH 7, respectively.

Amylase activity was recorded at different temperature value from lowest to highest such as at 30, 40 and 35°C, respectively (Figure 4). Therefore, the species identified from this range of temperature was *Pseudomonas* genera because the availability or growth favors *Pseudomonas* species with almost all surviving at 35 - 40°C. This is almost similar to the suggestion of Wang et al. (2016) in which the enzyme production reached maximum at temperature of 30°C, pH 7, with 40 g/L starch in the medium inoculated with 1.4% v/v spore.

Table 4. Starch degrading index of isolated colonies (SDI).

Isolate code	Diameter of colony (cm)	Diameter of clear zone (cm)	SDI of RB
AJUMC1	1.5	2.9	1.933
AJUMC2	1.3	2.7	2.07
AJUMC3	1.6	2.5	1.56
AJUMC4	1.4	3.0	2.14
AJUMC5	1.6	2.8	1.75
AJUMC6	1	1.8	1.8
AJUMC7	1.8	3.0	1.66
AJUMC8	1.3	1.6	1.23
AJUMC9	1.9	3.3	1.74
AJUMC10	1.6	2.8	1.87
AJUMC11	1.5	2.6	1.73
AJUMC12	1.3	2.8	2.15
AJUMC13	2.4	3.6	1.5
AJUMC14	1.6	2.7	1.69
AJUMC15	1.6	2.5	1.56
AJUMC16	1.3	2.8	2.15
AJUMC17	1.6	2.4	1.5
BJUMC1	2.3	3.5	1.52
BJUMC2	1.75	2.4	1.37
BJUMC3	1.2	2.0	1.66
BJUMC4	1.5	2.8	1.86
BJUMC5	1.0	2.1	2.10
BJUMC6	1.2	1.9	1.58
BJUMC7	1.2	2.25	1.875
BJUMC8	1.7	2.8	1.64
BJUMC9	1.2	1.9	1.58
BJUMC10	0.95	1.7	1.79
CJUMC1	1.5	2.6	1.73
CJUMC2	1.4	2.3	1.64
CJUMC3	1.1	1.9	1.72
CJUMC4	1.2	1.8	1.50
CJUMC5	1.8	2.9	1.61
CJUMC6	1.25	2.3	1.84
CJUMC7	2.5	3.6	1.44
CJUMC8	1.8	3	1.67
CJUMC9	1.2	2.1	1.75
CJUMC10	1.3	2.3	1.77
CJUMC11	1.6	2.9	1.81
DJUMC1	2.5	3.5	1.40
DJUMC2	0.8	1.2	1.50
DJUMC3	0.8	1.4	1.75
DJUMC4	1.7	2.7	1.59
DJUMC5	1.2	2.4	2.00
DJUMC6	1.7	2.5	1.47
DJUMC7	1.1	1.9	1.72
DJUMC8	0.9	1.8	2.00
DJUMC9	1.8	2.6	1.44
DJUMC10	0.84	1.3	1.57
DJUMC11	2.1	3.0	1.4
DJUMC12	1.4	2.3	1.64
DJUMC13	1.6	2.6	1.625
DJUMC14	1.2	1.6	1.33
DJUMC15	1.40	2.00	1.43



Figure 2. Amylase activity analysis on Petri-dish.

Table 5. Determination of different parameter.

Isolate code	pH (7)	Temperature (35°C)	Starch con. (1.5%)	Incubation period (24 h)
AJUMC	+	+	+	+
BJUMC	+	+	+	+
CJUMC	+	+	+	+
DJUMC	+	+	+	+

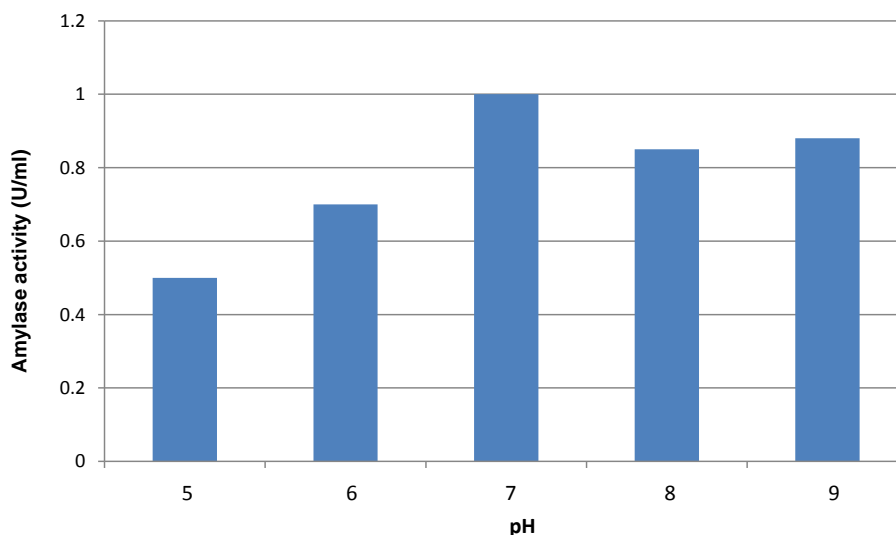


Figure 3. Amylase activity at different pH.

Conclusion

The present study reveals that all four sites soil samples taken from Jimma University plant root were inhabited

with diverse microorganisms mostly of *Bacillus* and *Pseudomonas* genera with high potential to degrade starch, thereby degrading starch blended material (polymers) to avoid pollution from the environment. The

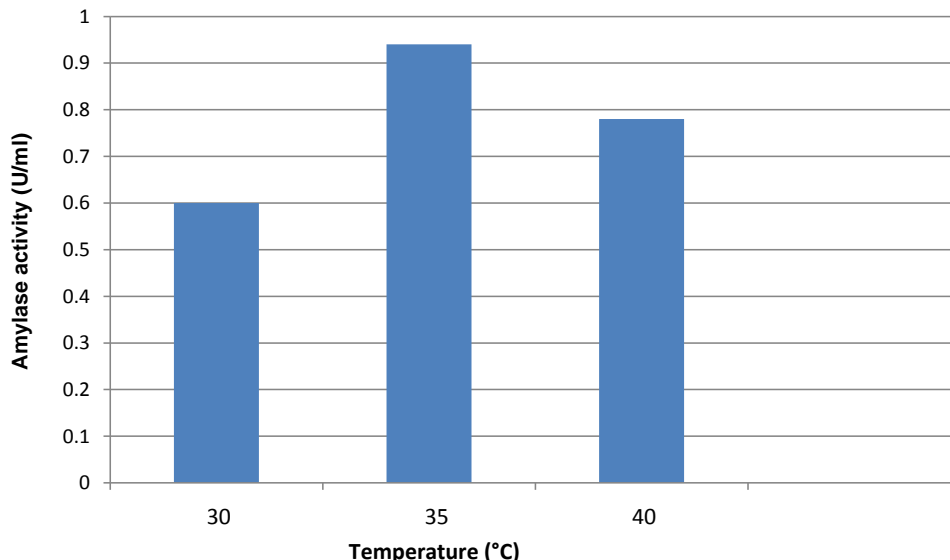


Figure 4. Amylase activity at different temperature.

result from Table 4 clearly indicates that the highest to lowest range of starch degrading index of rhizobacteria capacity was found from 2.15 – 1.23 cm range. Although amylase can be acquired from many plants and animals, microbial amylase generally meets industrial demand. Amylase activity was dramatically enhanced by Co^{2+} addition and slightly increased by Na^+ and Mn^{2+} addition. Bacterial isolate produced amylase at alkaline culture conditions with different factors greatly regulating the growth and production of amylases. The results in this study, on different factors, will be useful during further production of amylase by these microorganisms and the production of amylase depends on the microorganisms and the geographical area of the study design. Therefore, this is almost the best result and can be a key view for further study and commercialization. Based on the result of this study, further study must be carried out using a molecular identification system.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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