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ISOLATION AND CHARACTERIZATION OF AMYLASE PRODUCING BACTERIA FROM SOIL IN DEBRE BERHAN ETHIOPIA

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ABSTRACT

Amylase enzyme is an enzyme that hydrolyzes starch into smallest substance such as glucose. Debre Berhan is rich in numerous important microbes which can be used for production of different products with desirable industrial applications. This study aimed to isolate and morphologically and biochemically characterize the bacteria species and to optimize the growth parameter. The amylase producing bacteria were isolated from farmland around Debre Berhan, Ethiopia and characterized. For this study six bacterial strains (S1b2, S1b3, S2b2, S2b3, S3b2 and S3b3) were isolated and screened for amylase production through using iodine test on SNA and by measurement of OD value. Isolate S3b2 was resulted higher clear zone formation and OD absorbance of crude enzyme after enzyme production. Based on these characters the S3b2 isolate was selected for further process. Different biochemical and morphological tests such as gram staining, catalase, starch hydrolysis, VP, methyl red, endospore staining, motility and citrate utilization tests were done to characterize and identify the selected isolate. Finally, the selected isolate was identified as bacillus spp. The growth condition optimizations were performed to know the effect of different parameters such as temperature, pH and incubation period on bacterial cell growth. Accordingly, the optimum growth temperature, pH, incubation time was identified as 37^0c, pH 7 and 36 hrs respectively.

Key words: amylase, microorganism, starch degradation, clear zone

INTRODUCTION

Enzymes are biological catalyst, useful product from micro-organisms, animals and plants and used for the human demand (1). Microbes have become progressively important as producer of industrial useful enzymes. Due to microbial biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being faced to replace enzymes, which traditionally have been isolated from animal and plants (2). There are many processes at industrial aspect where enzymes play an important role (1). Amylase,

protease and lipase are among commonly used enzymes in industry, environmental and food biotechnology. Amylase is an enzyme that hydrolyzes starch into smallest substances such as glucose. During hydrolysis of starch by amylase enzyme glycosidic bonds are broken down and monomeric substances released. Such a like reaction is common in many existing creatures for producing and storing energy. Hereafter amylases are very predominant enzymes created in nature by numerous kinds of organisms such as plant, animal, human and microorganisms (3). Amylases obtained from microorganisms have been used by numerous industries as a source of enzyme for manufacture of foods, beverages, paper, textile pharmaceutical, cosmetic, fuel industries, and detergent industries. In general, enzymes manufactured from fungi and bacteria have several applications in different industries. Recently advancement in biotechnological tool, application of amylase enzyme has increased in clinical research, medical and starch analytical chemistry (1).

Amylases are produced endogenously in many different microorganism e.g. *B. subtilis, B. stereothermophilus, B. licheniformi, Aspergillus niger, A. orzyae, Pseudomonas* and *Clostridium,* in addition produced from animal organ and many plants. But bacteria are generally used for the production of amylase that used for starch hydrolysis. One of attracting reasons for using bacteria to get amylase is that bacteria are cost effectiveness, consistency, less space and time required for manufacture, easy to propagate and handle under controlled environmental factors like pH and temperature (4).

Among industrially important enzymes, amylases sharing 25-30% of world enzyme market are drawing more attention because of their wide commercial applications and economic benefits for producer and company which buy an enzyme for different application. A varied array of industries like foods, textiles, beverage and garments industries including clinical and medicinal chemistry use amylase for making their byproducts. They hydrolyze starch to generate maltose and maltotriose from amylose, while glucose, maltose and limit dextrin from amylopectin (5). This needs a continuous making of amylase enzyme. production of a mass amount of enzyme right from mother nature is impossible and henceforth several approaches are being continuously recognized to advance the bulk manufacturing of marketable amylase according to Tasnia (3). Several types of microorganisms were discovered and selected as a source for production of amylase because of their easily availability and simplicity of the process. Amylases sourced from fungi have been used throughout the world along with diverse strains of bacteria. Every bacteria strain needs specific optimum growth conditions and nutrients to yield amylase according to their environmental conditions. Bacteria inhabit different environment from area which living organisms inhabit to harsh environment such thermophiles and psychrophilic area. Soil is the main home of these bacteria that might be isolated and commercially grown in massive figures to manufacture an enormous quantity of enzyme. In order to offer this, many industries practice shake flasks fermentation to culture bacteria. Additionally, the produced enzymes need ideal environmental conditions for their high activity. This consists of factors such as temperature and pH. Therefore, finding out the ideal environmental conditions for amylase enzyme activity through research before it can be applied for industries is very important tusk (6).

Amylases are classified in to endo-acting, exo-acting, and debranching enzymes. Among the amylases, α-amylase is endo-acting whereas β-amylase is exo-acting enzyme. There were various reports on starch degrading microbes from different sources. Soil that enriched with starchy material is one of the rich sources of starch degrading microorganism as it contains mostly starchy substrates (2). This research was done by isolating of amylase producing bacteria, from soil samples collected from barley farm and physiological and biochemical features were determined.

Some researchers have reported amylase producing bacteria from diverse environmental habitats such as geothermal sites, starch material disposed sites such as wheat farm, limen pale and hot spring sites. The state of Debre Berhan which is found in Amara regional state of Ethiopia is also rich in barley and wheat farms which are expected to have starch degrading microbes. The microbial diversity of barley and wheat farms and other waste material has not yet been fully studied in Ethiopia, but there are few sporadic reports in amylase producing bacteria and actinomycetes by gebreselama (7). Therefore, the aim of this study is to isolate and characterize amylase producing bacteria from barley farm Sediment samples. Microorganism which are found in starch rich soils such as barley and wheat farm have many applications in industries, biotechnology and industrial biotechnology in terms of their enzyme extract (8).

MATERIAL AND METHODS

Description of the study area

The study was carried out in Debre Berhan University biology laboratory. Debre Berhan is a Town found in Amara regional state of Ethiopia. The Town is located in the Northern of Addis Ababa and found 120 km far from capital city of Ethiopia.

Materials

Materials and tools that used in this research were namely conical flasks, glass pipettes, vortex, dropper, beakers, glass rods, inoculation needle, autoclaves, petriplates, spectrophotometer, PH meter, electronic balance, measuring cylinder, incubator, laminar air flow hood, water bath, refrigerator and washing bottle. These materials and tools supplied from Debre Berhan University biology research laboratory.

Sample collections

Different soil samples were collected from six sites of barley farm in Debre Berhan. These samples were collected by using sterilized bottle and brought to Debre Berhan University biology laboratory and incubated in the refrigerator until next isolation of bacteria.

Isolation of bacteria from soil samples

Soil samples that collected from three different sites were homogenized by mortar and pestle. Isolation of bacteria from soil was done through preparation of serial dilutions to get the appropriate microorganisms from each sample. From soil sample 1g was transferred into 9 ml of distilled water containing 1% of peptone and homogenized by vortexing. 1ml of sample from the first test tube was measured and transferred into the second test tube containing 9 ml of the same amount of water. It was carried out up to 10^{-6} for each soil sample. Accordingly, 0.1 ml solution was taken and inoculated by using spread plate technique on nutrient agar medium in triplicate. The cultures were incubated at 37^0c for 24 hrs (9).

Preparation of pure culture

From mixed total bacteria single colonies were selected and inoculated on fresh nutrient agar medium by streak plate technique to get pure culture bacteria (10).

Evaluation of the isolates for amylase production

Bacterial colony from pure culture were inoculated into new starch nutrient agar medium containing

nutrient agar in triplicate and incubated at 37^0c for 24 hrs. The growth of bacteria in the starch nutrient agar media was observed and after 24 hrs the culture was flooded with iodine solution to examine amylase enzyme production status. The positive results were known by clear zone formation around bacterial inoculum. The diameters of clear zone were measured by ruler (6).

Screening of amylase producing bacterial using starch nutrient broth

A loopful of bacteria from pure culture was taken and inoculated into starch nutrient broth (5 g/l of soluble starch, 13 g/l of nutrient broth). The broth was incubated at 37°C for 24 hrs with control group without inoculum. After 24 hrs of incubation 3ml of broth was taken and transferred in to test tubes from each sample of bacteria. The test tubes were centrifuged to separate enzyme from other media component. The precipitate was treated with acetone and incubated at -18°C for 2 hrs. Then it was centrifuged to separate acetone and 2 ml of %soluble starch were added into the precipitate. 1ml of acetate was added to stop pH shift and incubated at 37°C for 10 min. in water bath. Then 2 ml of DNase to obtain color change and incubate at 100°C for 5 min. The OD of each organism and control was measured by spectrophotometer (11).

Characterization of growth of bacteria

- **Effect of pH on bacterial growth**

To study the effect of pH on bacterial growth starch nutrient broth was prepared and 50 ml of starch nutrient broth was transferred into 5 different flasks. The pH was adjusted in such that pH 6, pH 7, pH 8 and pH 9. Loopful of inoculum were inoculated on each broth. The broth was stored at 37°C for 24 hrs. Then after 24 hrs the OD of each broth or each pH was measured at 600 nm, the absorbance for growth. The broth with maximum OD value indicated optimum pH (12).

- **Effect of Temperature on bacterial growth**

To study the effect of temperature on bacterial cell growth starch nutrient broth was prepared and 50ml of starch nutrient broth was transferred into 5 flasks. The inoculum was incubated at 30, 37, 40 and 45°C for 24 hrs. The OD of broth incubated at each temperature was measured at 600nm, which is absorbance for growth (13).

- **Effect of Incubation Period on bacterial growth**

To study the effect of incubation period on bacterial cell growth amylase producing bacterial growth was determined by adding selected bacteria in the production media. The experiment was carried out by incubating bacteria at different incubation periods such as 24, 36, 48 and 72 hrs. The growth of bacteria was checked at each incubation time by measuring OD at 600 nm (13).

Morphological Characterization

The macroscopic and microscopic appearance of isolates was observed by necked eye and microscope. The isolates were observed by light microscope after stained with gram's reagent. The shape of bacteria and its grams status whether gram negative or positive were examined. Endospore staining was carried out to know whether the bacteria are spore former or vegetative. Various physiological and morphological analyses were used to detect the inoculum according to Bergey's Manual of Determinative Bacteriology.

Biochemical Characterization

The biochemical tests such as citrate utilization test, catalase test, methyl red test, and motility test were carried out. Starch hydrolysis test were carried out to check whether the bacteria produce amylase or not.

Analysis of the data

All the data were analyzed and organized by using Microsoft excel spread sheet, table and photograph and graph. Finally, these data were summarized according to the result and analysis methods.

RESULT AND DISCUSSION

Isolation of bacteria from soil sample

Three soil Sediment samples were taken from barley farm around Debre Berhan. Total bacteria were grown by serial dilution method and spread method on nutrient agar by spread plate technique and incubated at 37°C for 24 hrs. The mass of bacteria was grown on a sample that taken from 10^{-3} dilution factor and mixed population of bacteria was resulted.

Preparations of pure culture bacteria

The pure culture bacteria were grown on NA medium by streak plate technique and stored at 37°C for 24 hrs (Figure 1). After 24 hrs incubation 6 isolates of pure culture were selected and cultured on starch nutrient media (7)

Figure 1: Pure culture of amylase producing bacteria

Screening Amylase-producing bacteria

The bacteria were grown on a nutrient agar medium contains 5% soluble starch, incubated at 37°C for 24 hrs. Amylase producing bacteria was successfully screened from the bacteria isolated from the pure culture. This condition was observed from the formation of a clear zone around the colony

with the iodine test in starchy medium (Figure 2). A clear zone showed that the starch was hydrolyzed by the amylase enzyme produced from bacteria; whereas purple zone was the results of starch and iodine reaction. Two isolates (s1b2 and S2b3) were indicating maximum clear zone formation and selected as high amylase producer (Table 1).

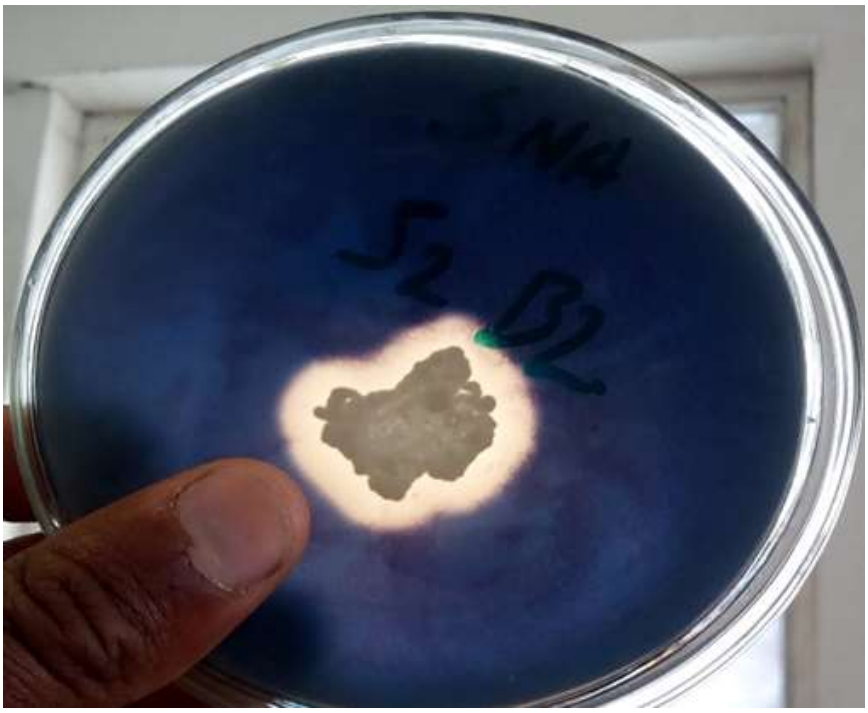

Figure 2: Clear zone around the colony with the iodine test in starchy medium

Table 1: Measurement of clear zone

Isolate	Clear zone (cm)	Inoculum size (cm)	Ratio (cm)
S1b2	3	1.5	2
S1b3	2	1.6	1.25
S2b2	1.8	1.5	1.2
S2b3	2.3	1.7	1.3
S3b2	1.7	1.2	1.4
S3b3	1.6	1.1	1.3

Fermentation test was carried out to select the best amylase producer from the two isolates. Starch nutrient broth inoculated with test organism was incubated for a purpose of starch hydrolysis. Crude enzyme was prepared by centrifugation of the broth at 3000 rpm for 10 minutes. 2 ml of soluble starch was added and then 1ml of DNase was added after the crude enzyme and starch were reacted to obtain color change. The best color change was observed in isolate s3b2 (Figure 3). The OD of the two isolates was measured after it was reacted with soluble starch.

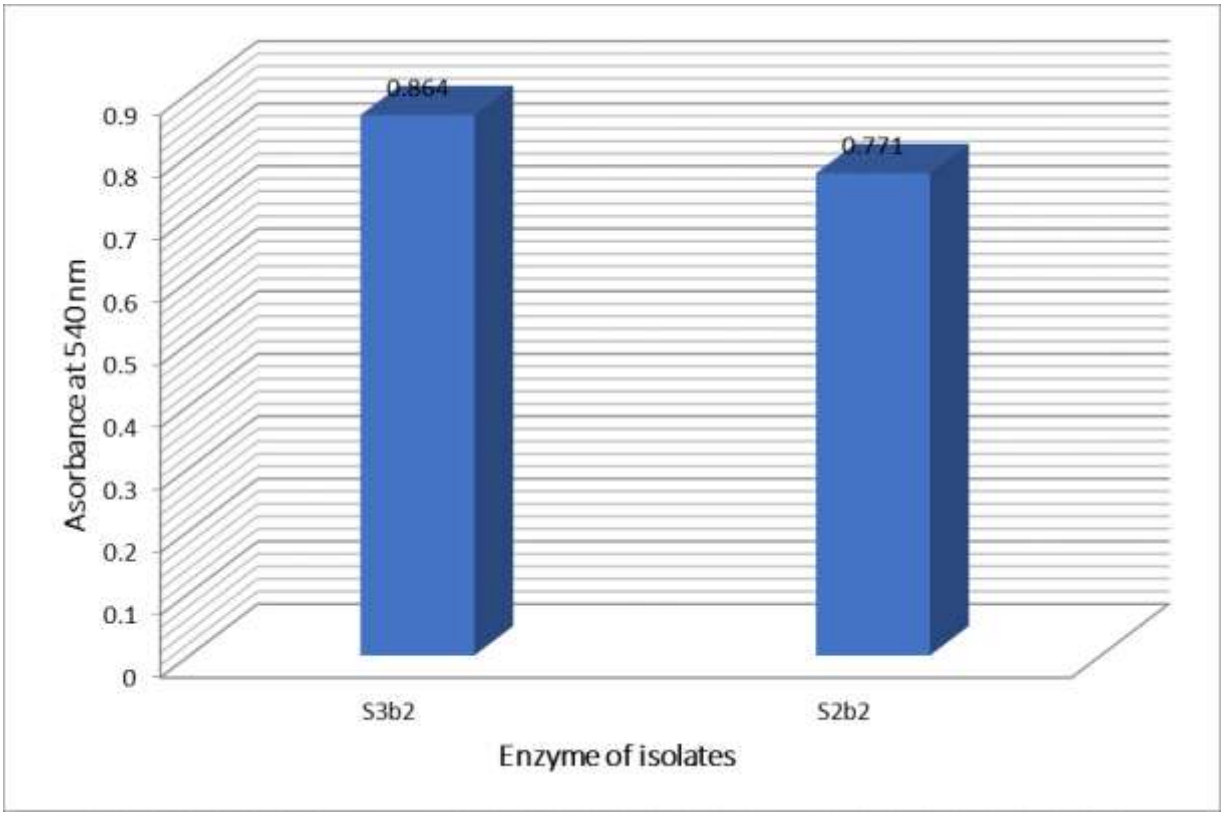

Figure 3: OD of enzymes at 540 nm

The genus of the amylase-producing bacteria was identified by observation of the morphology and some biochemistry tests described below (Table 2). After amylase producing bacteria identification had been performed, the growth parameter of amylase producing bacteria was optimized.

Characterization of growth conditions of bacteria

- **Effect of PH on bacteria growth**

Bacterial inoculums were inoculated on starch nutrient broth containing (13g/l nutrient broth, 5g/l soluble starch). The medium pH was adjusted into 6, 7, 8 and 9. Each inoculated media were incubated at 37℃ for 24 hrs for growth. The OD was measured for each pH variable at 600 nm, absorbance for microbial growth (14). The OD of each test was recorded as shown in figure 4. The maximum absorbance was recorded at pH7.

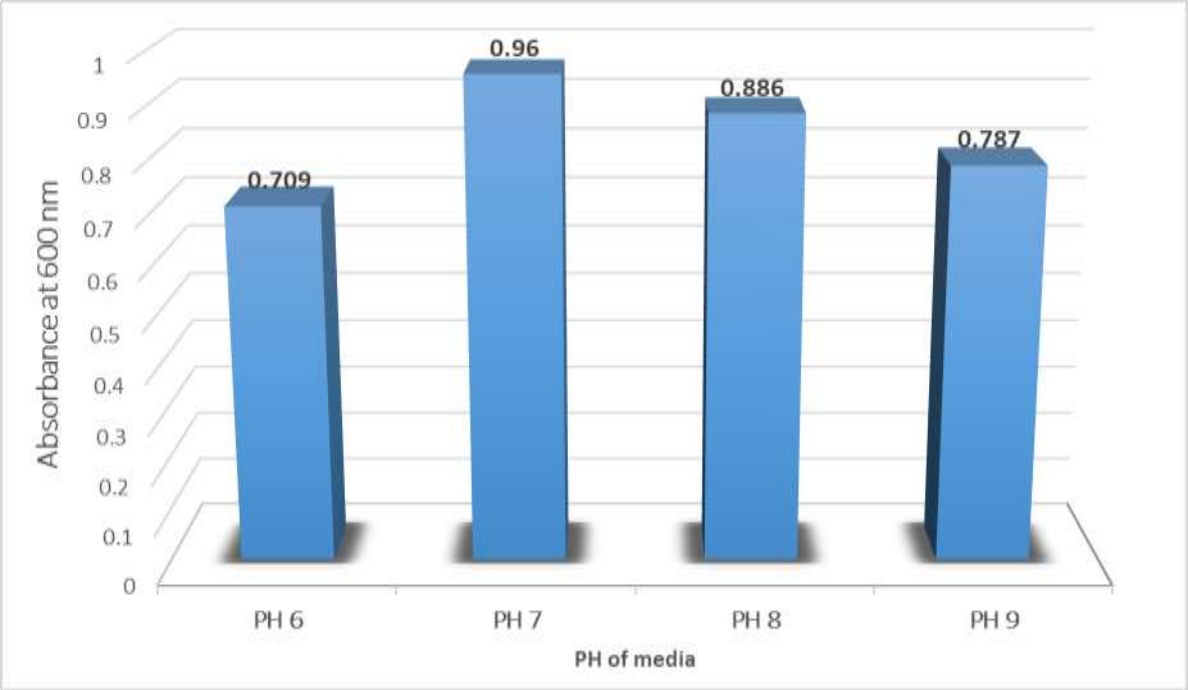

Figure 4: Effect of PH on bacteria growth

- **Effect of temperature on bacteria cell growth**

To evaluate effect of temperature on bacteria cell growth the starch nutrient broth containing (13g/l nutrient broth, 5g/l soluble starch) was prepared and it distributed into 3 flasks and incubated at variable temperature (30, 37, 40, 45℃) for 24 hrs. After 24 hrs growth the OD of each variable was measured (14). The maximum absorbance was at 37℃. The absorbance of each growth temperature was recorded (Figure 5).

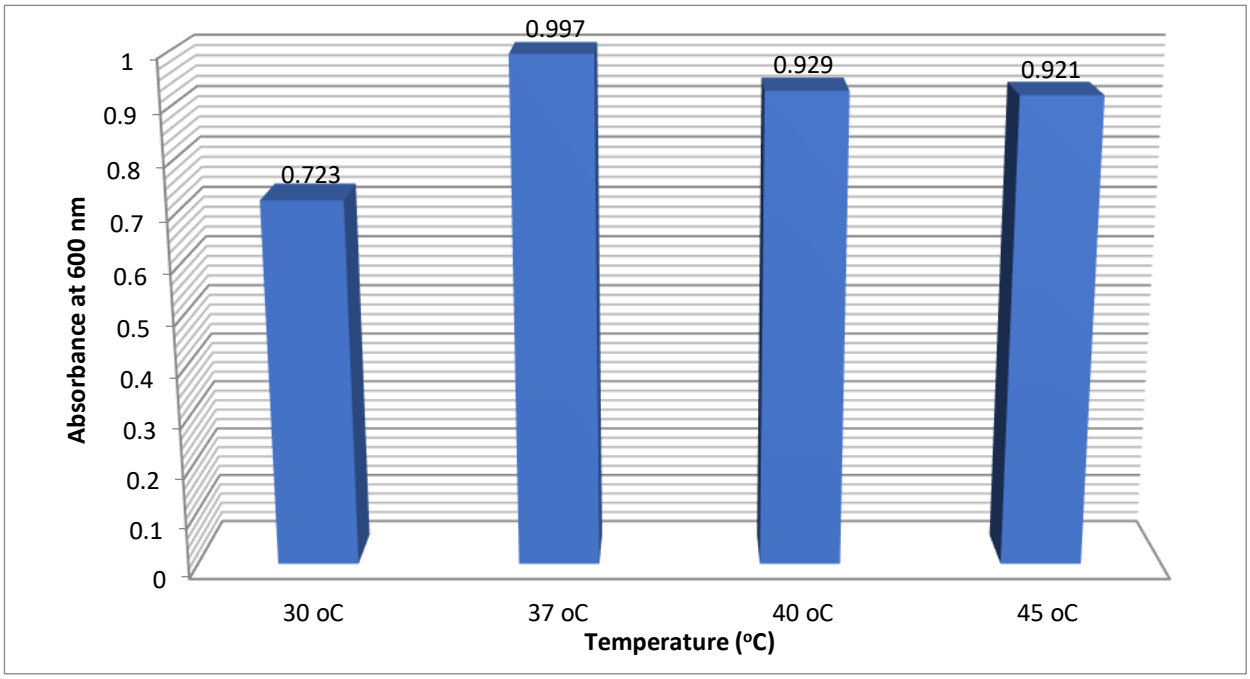

Figure 5: Effect of temperature on bacteria cell growth

- **Effect of incubation period on bacteria growth**

The inoculum was inoculated on a starch broth and stored for 24, 36, 48, and 72 hrs at 37 ºC. The growths of culture were checked at each incubation time its OD was measured (14). The maximum bacterial growth was observed at 36 hrs incubation. The optical density of the culture at each incubation time was recorded (Figure 6).

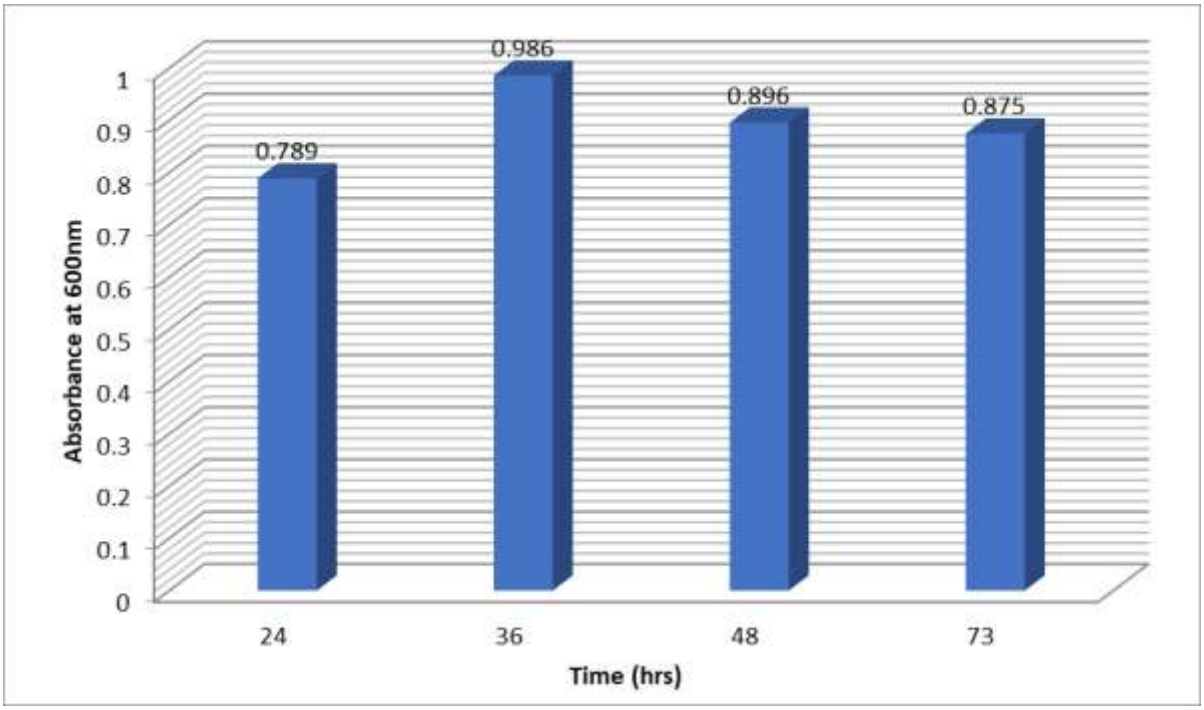

Figure 6: effect of incubation time on bacterial growth

Physiological and biochemical characterization of amylase producing bacteria

- **Morphological characterization**

Isolated bacterial colony was macroscopically and microscopically observed and resulted in white colored, medium sized, rounded. Morphological test of gram staining showed gram positive, rod shaped. Endosperm staining showed that the isolate were spore former. The isolate showed positive result for growth on 6.5% NaCl at 37ºC.

- **Biochemical Characterization**

Various biochemical tests including catalase, motility, fermentation and citrate utilization, MR-VP, and starch hydrolysis tests were done to identify bacteria. The isolate indicated positive for catalase test, citrate utilization test, Starch Hydrolysis test and motility test (Figure 7b). It was negative result for methyl red test (Figure 7a).

Table 2: Biochemical characterization of bacteria isolates

Isolates	Gram staining	Catalase test	Starch Hydrolysis test	VP	Methyl red test	Endospore staining	Motility test	Citrate test
Isolate 1	+	+	+	+	-	+	+	+
Isolate 2	+	+	+	+	-	+	+	+

Figure 7: a) result for methyl red test b) results of citrate utilization test

Discussions

Amylase producing microorganisms can be extracted from different habitats such as farm lands, water sources, fruits and vegetable processing sits, etc. But soil is known to be the main source of amylase producing microorganisms and is sometimes considered as the store of amylase producing microorganisms. In this study amylase producing bacteria were isolated from barley farm soil. The isolation of these organisms was accomplished through using serial dilution and spread plate method. Comparable technique had been used by Padma sigh and pallavi kumari (6). The isolated bacteria were predominantly selected for the production of amylase through using starch agar plate technique. Then these organisms were evaluated for production of amylase by the starch hydrolysis test (15). Almost all isolates were amylase producer with varied production status. The selection of best amylase producer bacteria was done by measuring clear zone diameter that is hydrolyzed by bacteria and by starch hydrolysis test using measuring OD. The best or higher amylase producing bacteria was found with higher clear zone formation and high value of optical density. Low amylase

producer was those bacteria with low clear zone formation on starch nutrient agar and low OD value (15)

The isolate was characterized morphologically and biochemically. The bacterium identified based on burgey's manual of determinative bacteriology. The bacteria were gram positive, rod, and spore former and was grown in 6.5% NaCl at 37ºC and showed negative result for growth at 55 ºC. It was positive for biochemical tests such as citrate utilization, catalase, VP and motility test. But it indicated negative result for methyl red test. According to burgey's manual the isolate identified as *B. subtilis*. This identification was similar with the previous report by Hansa Singh (11).

Temperature and pH are among critical parameters for growth of amylase producing bacteria. Temperature is one of the other factors influencing growth of amylase producing bacteria. The OD of the bacterial growth was recorded at different temperatures revealed that bacteria show maximum growth at 37ºC (Figure 5). Microbial growth was increased with increasing incubation temperature until it reaches to the optimum and then progressively decreased with further increase in incubation temperature. Similar result was reported by Karnwal and Nigam, (13). The pH for growth of the organism was tested by using OD measurement like that of temperature and the maximum growth observed at pH 7. Similar result reported by Soumya Vaidya and Pragya Rathore (16). The incubation time for bacteria growth was maximum at 36 hrs. The growth of bacteria increased gradually up to 36 hrs incubation times and decreased gradually with further increase in incubation period.

CONCLUSION

In this study, an attempt has been made to isolate amylase producing bacteria from soil Sediment sample. A total of two bacterial isolates which produced clear halos in the starch- nutrient agar medium were isolated and purified. One best amylase producer bacterial isolate was isolated based on clear zone on starch nutrient agar ad OD. The isolate was gram positive, rod, spore former bacteria. Biochemical characterization of the selected bacterial isolates was done; the isolates showed positive results with, Citrate test, catalase test, urease test and Starch hydrolysis. From these results, the isolate was identified as Bacillus sp while negative for methyl red test (MR). According to burgey's manual the isolate was bacillus spp. The optimum temperature, incubation period and pH for growth of amylase producing bacteria were 37°C, 37 hrs and 7 pH respectively.

Recommendation

Based on the result of the study the researcher would like to recommend that:

The molecular characterization of this bacteria should be further studied

Biochemical tests such as vp test, should be tested

Effect of carbon concentration, metal ion effect and substrate concentration should be studied.

Ethics approval and consent to participate

The author would like to approve that this submission has no ethical related material and all data and included material is not ethics related.

Consent for publication

The author would like to confirm the agreement for this publication.

Availability of data and material

The author would like to confirm that all required data included for this publication.

Competing interests

The author would like to confirm that there is no conflict of interest on this publication.

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