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Isolation and Identification of the Thermophilic Amylase-Producing Bacteria from Soil of North of Iran

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Soil samples were screened from amylase- producing bacteria on starch agar medium (Nutrient Agar containing 1 % soluble starch). Amylase-producing bacteria was identified by clear zones of starch hydrolysis around colonies by flooded with lugol solution. Starch containing areas are coloured dark blue,while areas around the colonies hydrolysis starch were clear,showing the effect of extracellular amylase that had broken the bonds in the starch. 31 Thermostable amylase producing bacteria were isolated from different soil samples (Compost, Hot spring and Manures) collected from various parts of North of Iran. These bacteria were examined for various morphological and biochemical characteristics according of Bergey's manual of determination bacteriology. The best starch degrader were *Bacillus* species.

Key Words: Amylase, Thermophilic bacterium, *Bacillus* sp, Soil, Isolation, Hydrolysis.

INTRODUCTION

Several intensive studies were performed to isolate thermophilic carbohydrate-fermenting bacteria from several habitats, aiming to use of these microbes and their enzymes for biotechnological applications[1-3]. Microbial enzymes are used in the backing, brewing, distilling, starch and textile industries.The majority of the investigated thermophilic bacteria belongs to the genus bacillus have been isolated from thermophilic and mesophilic environments[3-5].

Some thermostable α-amylase producing bacteria were isolated from different soil samples collected from various part of Iran and identified[6]. Amylases are among the most important enzymes used in biotechnology, particulary in process involving starch hydrolysis.

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Though amylase originated from different sources (plant, animals and microorganisms), the microbial amylases are the most produced and used in industry due to their productivity and thermostability[7]. Natural fermented media (foods and soils) offer the substrates for isolation of microorganism strains producing amylases. In this respect, many strains used in food industry originate from fermented food media, while soil, particulary wastes and mud offer strains used mainly in chemical industry[7-9].

Amylases of microbial orgin have found application in the starch processing industry for the production of chemicals, sweeteners and syrups and for the preparation of media components for use in microbial fermentation[10].

Based on their activity with starch substrate, they have been classified as α-amylases (E.C.3.2.1.1), glucoamylases (E.C.3.2.1.1), β-amylases (E.C.3.2.1.2), isoamylases (E.C.3.2.1.68) and pullulanases (E.C.3.2.1.41).

Though each amylases has a specific role in starch processing. A combination of enzymes has been generally recommended for the industrial processing of starch. The production of economically important α-amylase is essential for the conversion of starch into oligosaccharides. This enzyme is extensively used in starch liquefaction, paper industries, food, pharmaceutical and sugar industries[10,11].

Although α-amylase, an example of endo-type enymes are widely distributed in various kind of organisms. β-Amylase are produced only by plant and some Gram-positive spore forming bacteria such as *Bacillus cereus*[12], *Bacillus polymyxa*[13], *Bacillus circulans*[14] and *Clostridium thermosulfurogenes*[15,16].

Amylase are important enzymes employed in the starch processing industries for hydrolysis of polysaccharides such as starch into sugar constituents[17], starch degrading enzymes like amylase received great deal of attention because of their technological significance and economic benefits. Evidences of amylase in yeast, bacteria and moulds have been reported and their properties documented[17-19]. In recent years the new potential of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms[17,19,20].

Bacteria α-amylase have several applications in the food industry and are potentially useful in the pharmaceutical and some chemical industries[21-26]. The starch processing industries require the use of amylolytic enzymes at high temperatures[27,28].

Thermophilic organisms are therefore of special interest as a source of novel thermostable enzymes[29-31]. The advantages for using thermostable α-amylases in industrial processes include the decreased risk of contamination, the increased diffusion rate and the decreased cost of external

cooling[26]. With the more wide spread use of α-amylase it has become essential to isolate new type producing microbial strains. Almost all microorganisms of the Bacillus genus synthesized α-amylase, thus this genus has the potential to dominate the enzyme industry[32].

The present study deals with the isolation and characterization of a moderately thermophilic Bacillus produce amylase. In this work, the isolation and characterization of amylase producing *Bacillus* sp, from soil which might have useful industrial applications, is reported.

EXPERIMENTAL

Screening, isolation and identification of microorganisms soil samples from different parts of North of Iran (including soils cattle and birds composts manure, plants composts and hot spring) were collected. Suspensions were prepared by mixing 10 g of each samples with 90 mL of sterile distilled water and were shaken vigorously for 2 min. The samples were heated at 60ºC for 1 h in water bath, the liquid was serially diluted in sterile saline and the dilution from 10^{-1} to 10^{-7} was poured and spread on to nutrient agar using spread plate and pour plate methods. Nutrient agar containing (g/L) agar, 15; yeast extract, 2; peptone, 5; sodium chloride, 5.

These plates were placed in plastic bags to reduced evaporation and incubated at 55ºC for 24 h[33]. Some of the colonies that grew on the plates were purified by successive streaking on nutrient agar plates.

Pure culture were maintained on nutrient agar slant stored in a refrigerator and subcultured at 3 month interval. All colonies isolated by this procedure proved to be thermophilic and did not grow in nutrient agar medium at or below 44ºC for as long as 7 d.

The pure isolated bacteria were then transferred onto agar plate containing 1 % soluble starch (g/L) agar, 15; yeast extract, 2; peptone, 5; sodium chloride, 5; starch, 10, to screening for amylase producing bacteria. The plate were incubated for up to 2 d at 55ºC after which the plate were stained with Gram's iodine solution (0.1 % I_2 and 1 % KI). The colonies with clear zone were picked up isolated for further investigation these were subculture to obtain pure isolated bacteria species using methods[34].

Evalution of clear zones of each colony was estimated as radius (mm) of the clear zone minus the radius of the colony. The diameter of the clear zone represented the amylolytic activity of strains (Table-2). The most active strains was used for former characterization. Properties of the isolated bacteria were identified based on cellular and colonial morphology, growth condition, gram stain, motility and biochemical tests[35,36].

In the identification of isolated bacteria species, standard taxonomic descriptions from[35] were used and *Bacillus* species were identified (Table-1).

TABLE–1
MORPHOLOGY AND CHARACTERISTICS OF
SOME THERMOPHILIC ISOLATES

Isolate code	Origin	Cellular morphology	Gram reaction	Spore test	Motility	Growth on Macconky	Growth on 7 % NaCl	Glucose	Lactose	Sucrose	Mannitol
Jin A	Plant manure	Rods	+	–	+	+	–	+	–	–	–
Jin B	Plant compost	Rods	+	+	+	–	–	+	–	–	–
Jin C	Animal manure	Rods	+	+	+	–	–	+	–	–	–
Jin D	Animal manure	Rods	+	+	–	+	+	+	+	+	+
Jin E	Water spring	Rods	+	+	+	+	+	+	+	+	+
Jin F	Water spring	Rods	+	+	–	–	+	+	+	+	+
Jin G	Plant compost	Rods	+	–	+	+	–	+	–	–	–
Jin H	Animal manure	Rods	+	+	+	+	+	+	+	+	–
Jin J	Water spring	Rods	+	+	–	+	+	+	–	+	+
Jin K	Plant compost	Rods	+	+	–	+	–	+	+	+	–
Jin L	Plant compost	Rods	+	+	–	+	+	+	+	+	+
Jin M	Animal manure	Rods	+	+	–	+	+	+	+	+	+
Jin N	Animal manure	Rods	+	+	–	+	+	+	–	+	+
Jin O	Animal manure	Rods	+	+	–	+	–	+	+	+	–
Jin P	Water spring	Rods	+	+	–	+	+	+	+	+	+
Jin Q	Animal manure	Rods	+	+	–	+	+	+	+	+	+
Jin R	Animal manure	Rods	+	+	–	+	+	+	–	+	+
Jin S	Animal manure	Rods	+	+	–	–	–	+	+	+	–
Jin U	Plant compost	Rods	+	+	+	+	–	+	+	+	+

Production of crude amylase

The isolated strains were cultured on starch agar plate and the plates were incubated at 50ºC for 18 h. Liquid media (*ca.* 5 mL) was pipetted into the starch agar plates and the cells scraped off using a sterile Pasteur pipette.

Liquid media contains ($MgSO_4 \cdot 7H_2O$ (0.1 %), $FeSO_4$ (0.05 %), $(NH_4)_2SO_4$ (0.1 %), bacto-peptone (1 %), yeast extract (0.5 %) and soluble starch (1 %).

50 mL in a 250 mL Erlenmyer flask was inoculated with this suspension to give an initial absorbance at 470 nm of at least 0.1 and the culture were incubated at 50ºC with vigorous aeration in shaker at 250 rpm for 1 d. At the end of each fermentation batch the cells of culture were separated by centrifugation at 5000 g, the clear supernatant was used as crude enzyme preparation.

Amylase activity

A solution of 65 % (w/v) sodium sulphate was added to the clear supernatant and enzyme recuperated by centrifugation at 8000 rpm. The enzyme precipitated was then suspended in phosphate buffer 0.005 M pH 6. The crude extract was used to determined enzyme activity. Amylase activity was measured by 3,5-dinitro salicylic acid (DNS) method[37].

The reaction mixture contained 1 mL of 1 % soluble starch in an appropriate buffer (citrate-phosphate buffer ph 6.5) and 1 mL of culture extract enzyme solution, after incubation in a water bath at 40ºC for 0.5 h the reaction was stopped by adding 2 mL of DNS reagent (1 g of DNS, 20 mL of NaOH and 30 g of sodium potassium tartarate in 100 mL). The mixture was boiled for 5 min, after cooling, the reaction mixture was diluted with 16 mL of distilled water and the absorbance was measured at 540 nm.

RESULTS AND DISCUSSION

Screening of microorganisms

75 Thermophilic bacteria have been isolated from soil samples according to the method[33]. The isolated bacteria were gram-positive and gram-negative and of which 40 of isolated were identified as *Bacillus* species with centeral spores and predominatly unswollen cylindrical sorangia.

30 Of isolated of hydrolyse both starch and casein and 20 of which hydrolysis any starch and 25 of them hydrolysis casein, all of isolated were catalase positive, indole was not formed and acetoin formation was positive. no growth was obtained in nutrient broth containing more than 7 % NaCl.

All of them grew in nutrient broth at 30-60ºC with a optimal at 50ºC, on the basis of these morphological and Biochemical characteristic, isolated strain as (Table-2) were classified in the genus Bacillus[35] and denominated *Bacillus* sp. They were *B. brevis*, (5 strain) *B. licheniformis*, (4 strain), *B. subtilis* (4 strain), *B. polymyxa* (2 strain), *B. mycoides* (5 strain), *B. cereus* (6 strain), *B. megaterium* (8 strain), *B. alvei* (3 strain), *B. coagulans* (3 strain)[36], identification *B. subtilis*, *B. licheniformis*, *B. cereus* and *B. megaterium* in soil isolations, in another investigation[6] isolated some thermostable α-amylase produing bacteria from different soil samples collected from various part of Iran and identified as *B. coagulans*, *B. licheniformis* and *B. brevis*[38], 29 is isolated as *B. megaterium* and 24 isolates as *B. subtilis* out of 306 soil samples. One may agree with the results of this study that *Bacillus* genera are wide spread among bacteria in soil.

TABLE-2
BIOCHEMICAL AND BIOPHYSICAL TESTS FOR IDENTIFICATION OF THERMOPHILIC ISOLATES

Isolate code	Catalase	Oxidase test	Methyl red	Vogos Proskauer	Citrate ultization	Urease activity	Starch hydrolysis	Casein hydrolysis	Gelatin hydrolysis	No.3 reduction	Indole	Probable identify
Jin A	+	+	–	+	–	–	+	+	+	–	+	*B.alvei*
Jin B	+	+	+	–	–	–	+	+	+	–	–	*B.brevis*
Jin C	+	+	+	–	–	–	+	+	+	–	–	*B.brevis*
Jin D	+	+	–	+	+	–	+	+	+	+	–	*B.subtilis*
Jin E	+	+	–	–	+	–	+	+	–	–	–	*B.megaterium*
Jin F	+	+	–	+	–	–	+	+	–	+	–	*B.mycoides*
Jin G	+	+	–	+	–	–	+	+	+	–	+	*B.alvei*
Jin H	+	+	–	–	+	+	+	+	+	+	–	*B.cereus*
Jin J	+	–	+	+	+	–	+	+	+	+	–	*B.licheniformis*
Jin K	+	+	–	–	–	–	+	+	–	+	–	*B.coagolase*
Jin L	+	+	–	–	+	–	+	+	+	+	–	*B.megaterium*
Jin M	+	+	–	–	+	–	+	+	+	+	–	*B.megaterium*
Jin N	+	–	+	–	+	–	+	+	+	+	–	*B.licheniformis*
Jin O	+	+	–	+	–	–	+	+	–	+	–	*B.coagolase*
Jin P	+	+	–	+	+	–	+	+	+	+	–	*B.subtilis*
Jin Q	+	+	–	–	+	–	+	+	+	+	–	*B.megaterium*
Jin R	+	–	+	–	+	–	+	+	+	+	–	*B.licheniformis*
Jin S	+	+	–	+	–	–	+	+	+	+	–	*B.mycoides*
Jin U	+	+	–	+	+	–	+	+	+	+	–	*B.subtilis*

Few publications are devoted to the study of the *Bacillus species* isolated from various environment. Due to their ubiquity and capability to survive under adverse condition, heterotrophic Bacillus strains and hardly considered to be species of certain habitats[39].

The Bacillus genus is an extensive heterogeneous group encompassing 83 valid described species to date. Many species in this taxon one of major clinical importance, such as the *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides* and *B. weihenstephanensis*, but unfortunately, members of the group show a great deal of morphological and biochemical similarities[40,41].

Since the biochemical approach for species identification can be tedious, expensive and inaccurate, a rapid definitive method is greatly needed. Molecular procedures are increasingly being used for rapid species identification[42]. In contrast, the environmental and non-pathogenic species of this genus exhibit a wide range of physiology DNA base content, and nutritional requirements[41].

Screening for amylolytic activity

The amylase activity was assayed using starch-agar medium and expressed as diameter of clear zone in mm. Strain Jin E, F, L, P, S exhibited the highest amylolytic activity with a clear zone diameter of 60-65 mm. (Table-3).

Table-3, showed the quantitative assessment of amylase productivity by the isolates using fermentation process strains Jin E, L, P, S also produced the highest enzyme activity (806-820 U/mL). Hence it was selected for subsequent investigation. *Bacillus* species were selected for further studies.

TABLE-3
AMYLASE PRODUCING ABILITY OF ISOLATED STRAINS

Isolates code	Means of clear zones (mm)	Amylase activity (U/mL)	Isolates code	Means of clear zones (mm)	Amylase activity (U/mL)
Jin A	47	650	Jin L	62	811
Jin B	55	720	Jin M	55	780
Jin C	51	680	Jin N	28	300
Jin D	48	600	Jin O	45	605
Jin E	65	820	Jin P	61	810
Jin F	60	800	Jin Q	42	400
Jin G	42	525	Jin R	46	485
Jin H	50	750	Jin S	63	806
Jin J	34	350	Jin T	44	420
Jin K	38	450	Jin U	28	325

The occurrence of amylolytic organisms from the soil agrees with earlier reports[18,43] that the soil is known to be a repository of amylase producers. Most commercial amylase mainly have been reported produced from the genus Bacillus[6,44-49].

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