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# Quantification of Amylase Enzyme from the Calcite Residing Actinobacteria Isolated from Limestone Quarries of Deccan Traps

**Syed Raziuddin Quadri[1,2,3], Jamal Al Ruwaili[1], Shaik Mohammed Akbar[5], Wen-Jun Li[3,4] and Dayanand Agsar[2]**

[1]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Northern Border University, Arar-91431, Northern Borders, Saudi Arabia.
[2]Department of Microbiology, Gulbarga University, Gulbarga-585106, Karnataka, India.
[3]Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education and Laboratory for Conservation and Utilization of Bio-resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan, 650091, PR China.
[4]State Key Laboratory of Biocontrol and Guangdong Key Laboratory of Plant Resources, College of Ecology and Evolution, Sun Yat-Sen University, Guangzhou 510275, PR China.
[5]Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Telangana State, India.

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**Sixty three actinobacteria were isolated from Limestone quarries of Deccan Traps from South India. The isolates were identified by morphological, biochemical and physiological studies. Among these, six isolates were selected on the basis of their amylolytic activity using submerged cultivation. The potential isolates exhibiting amylolytic activity were DRQ10, DRQ20, DRQ86, DRQ33, DRQ41, and DRQ63 as confirmed by formation of clear zones of starch hydrolysis around the colonies, with maximum activities 2500, 1100, 1200, 1150, 1250, 1400, and 1600 Units, respectively, as determined by DNS (3, 5-dinitrosalicylic acid) assay method. Various physicochemical parameters such as pH, temperature, incubation time were optimized for the production of amylase. Amylase production was maximum on 4$^{th}$ day of incubation. The optimum pH and temperature were found to be 9.0 and 45 $^{o}$C, and NaCl concentration of 3M for the potential amylolytic isolates. The carbon and nitrogen source for the production of amylase was starch and peptone, respectively.**

**Key words:** Actinobacteria, Amylase, Limestone quarries, Deccan traps.

Actinobacteria are Gram-positive mycelium forming bacteria, capable of producing large variety of different antibiotics and enzymes. Actinobacteria have been isolated from marine[1], as well as terrestrial[2] sources. Actinobacteria with saprophytic existence occur greatly in natural and manmade environments. Most of them are strictly saprophytes, but some of them form parasitic or mutualistic association with plants and animals. Actinobacteria have become the subject of intensive searches for sources of new, biologically active compounds, such as, antibiotics, antitumor agents, enzymes, enzyme inhibitors and growth promoting substances. Actinobacteria are filamentous Gram-positive bacteria, characterized by complex life cycle. They belong to the phylum Actinobacteria, which represents one of the largest taxonomic units among the 18 major lineages, currently recognized within the Domain Bacteria[3]. They play crucial role in recycling refractory biomaterials by decaying complex mixtures of polymers in dead plants, animals and fungal materials. They are also important in soil biodegradation and humus formation as they recycle the nutrients associated with recalcitrant polymers, such as chitin, keratin, and

* To whom all correspondence should be addressed.
Tel +966 594624402, Fax: +966 146644090
E-mail: raziuddinquadri@hotmail.com

lignocelluloses[4, 5]. They produce several volatile substances like geosmin responsible of the characteristic wet earthy odour[6]. The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000, of which 10,000 are produced by actinobacteria, thus representing 45% of all bioactive microbial metabolites discovered[7].

Large quantities of microorganisms, such as Fungi, Bacteria, Lichen and Algae are seen in a variety of environments in karst areas and they participate in a number of geological and geochemical processes especially the sedimentation and weathering of rocks and minerals and the formation and evolvement of soils. Extracellular hydrolytic enzymes secreted by actinobacteria are too important and meriting studies[8]. With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Starch hydrolysis, catalyzed by α-amylase, is one of the most important large scale uses of actinobacteria in terms of enzymatic processes[9]. Many actinobacterial species have been studied for degradation of starch, hemicellulose, lignin and cellulose[10, 11, 12]. Amylases can breakdown complex sugars such as starch into simple sugars such as glucose, maltose and dextrin. Bacteria, Actinobacteria and Fungi secrete amylases to the outside of the cells to carryout extracellular digestion. α Amylase (endo-1- α-D-glucanohydrolases EC 3.2.1.1) have been widely applied in clinical and medicinal purposes as well as for analytical chemistry. Besides their applications in starch saccharification they are also used in the food, baking, brewing, detergent, textile, paper and distilling industries[13, 14]. Glucose and maltose-forming a-amylases are employed in alcohol fermentation and sugar syrup formulation, and malto-oligosaccharide-forming α -amylases in food processing[15, 16].

The Deccan Volcanic province (DVP) is one of the world's largest igneous provinces and perhaps the best studied continental flood basalt (CFB). It has a total exposed area of about half a million square kilometers, between latitudes 16° - 24° N and longitudes 70° - 77° E. In the northwestern, central and southern Indian peninsula, the approximate volume of the DVP is about 2 x $10^6$ $km^3$ and its estimated age is 64-65 Ma (Million years ago)[17, 18]. Limestone is naturally occurring mineral that consists principally of calcium carbonate[19, 20, 21]. The aim of the present study was to screen amylolytic actinobacteria from the limestone rocks and characterize the enzyme for its stability at harsh conditions like alkalinity and high temperatures to serve the purpose of industrial usage.

## MATERIALS AND METHODS

**Sample collection from the Deccan traps**

Soil samples were collected from limestone quarries around Gulbarga in Karnataka and Betamcherla, Kurnool in Andhra Pradesh, India[22].

**Isolation of calcite residing Actinobacteria**

Samples were screened for actinobacteria by following serial dilution plate culture technique employing starch casein agar and ISP – 2, 3, 6 and 7 media. The inoculated plates were incubated at 35 °C for 3 weeks. Plates were observed at every 24 hours for the growth of actinobacteria. After the completion of incubation period, typical dry powdery colonies were picked up from mixed colonies and sub- cultured on fresh medium to obtain pure cultures. The pure cultures were stored at 4 °C[22].

**Isolation of amylolytic Actinobacteria**

A batch wise bioprocess was carried out in 250 ml Erlenmeyer's flask containing 100 ml starch casein broth. After sterilization of the medium at 121 °C for 15 minutes, 1 ml inoculum with spore count $10^8$ per ml was added. The inoculated flasks were incubated on rotary shaker (180 rpm) at 45 °C for 72 h.

**Screening of amylolytic isolates by Starch Iodine plate assay**

96 hour cultures were centrifuged at 10,000 rpm, 100 µl of the supernatants were kept in starch casein agar plates in a well, made in the center with the puncture tube. Following incubation, the plates were flooded with Grams iodine solution and a zone of clearance around culture supernatant of test isolates against a dark blue stained lawn of starch interpreted as a positive result.

**Optimization of culture conditions**

The various parameters like pH (4.0 to 12.0), temperature (30 °C to 50 °C), incubation time (1 to 6 days), NaCl concentration (0-5 M), carbon (starch, sucrose, maltose, fructose, and xylose) and

nitrogen (casein, oatmeal, peptone, tyrosine, yeast extract) sources were optimized for the production of amylase for the potential actinobacteria isolated from the limestone quarries of Deccan traps.

**Determination of extracellular amylase activity**

α-Amylase activity was assayed by measuring the release of reducing sugars in a reaction mixture of 1.0 ml of the crude supernatant and 1.0 ml of 1% (*w/v*) soluble starch (Sigma, MO, USA) solution in 0.05 mol/L phosphate buffer (pH 7.0) incubated at 50 °C for 20 min. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method at 550 nm spectrophotometrically (Systronics 105)[23]. One unit (U) of α-amylase activity corresponded to 1 mmol of glucose equivalent released per minute under the assay conditions[24].

## RESULTS

**Identification of amylolytic isolates**

Screening of amylolytic actinobacteria is shown in Fig. 1. Although six isolates were prominent among sixty three actinobacteria that exhibited hydrolysis zones, the actinobacteria DRQ10 strain showed better growth and maximum production of α-amylase.

**Influence of incubation time, pH, temperature, NaCl concentration on the production of amylase**

Amylase production was maximum on 4$^{th}$ day of incubation. The optimum pH and temperature were found to be 9.0 and 45 $^{o}$C, respectively, and NaCl concentration of 3 M was required for the potential amylolytic isolates for the production of amylase (Fig. 2, 3, 4, 5).

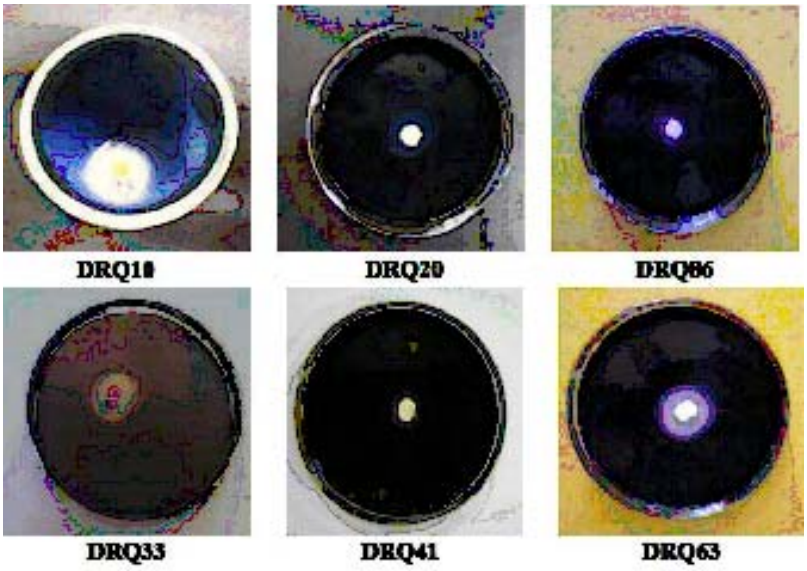


**Fig 1.** Synthesis of amylase by six novel isolates of actinobacteria

**Table 1.** The morphological characters of the selected actinobacterial isolates.

| Isolates | Aerial mycelium | Substrate mycelium | Diffusible Pigment | Sporulation pattern |
|---|---|---|---|---|
| DRQ10 | Greyish White | Greyish Brown | Greyish Brown | Flexuous |
| DRQ20 | Brown | Dark Brown | Brownish | Irregular Sporangia and mycelia |
| DRQ33 | Light Pink | Pink | None | Hooked |
| DRQ41 | White | White | None | Straight |
| DRQ63 | White | White | None | Straight |
| DRQ86 | Light Pink | Reddish Pink | None | Hooked /Curled |

**Effect of carbon and nitrogen source on the production of amylase**

The carbon and nitrogen source for the production of amylase was starch and peptone, respectively, for all the potential isolates (Fig. 6, 7).

## DISCUSSION

Actinobacteria have been of major scientific interest in the past decades, with the discovery of large number of metabolites produced by its diverse genera. The present investigation

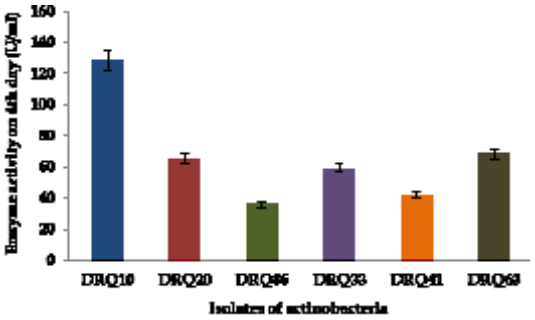


**Fig. 2.** Production of amylase by the novel isolates of actinobacteria isolated from the Deccan Traps

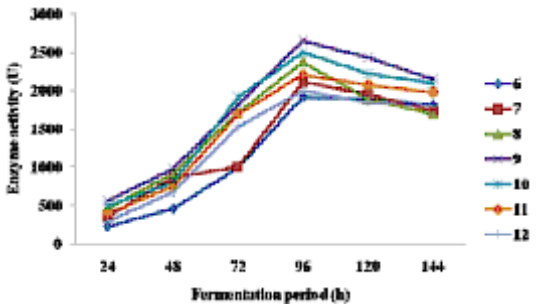


**Fig. 3.** Influence of pH on the production of amylase by the novel actinobacterium DRQ10

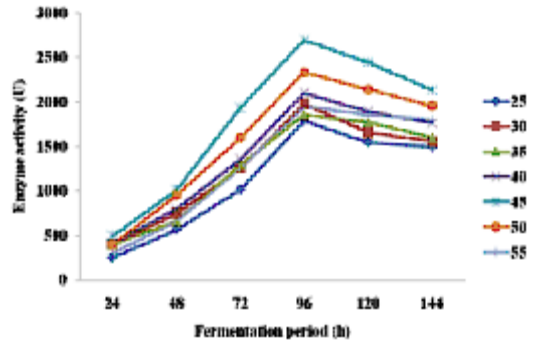


**Fig. 4.** Influence of temperature on the production of amylase by the novel actinobacterium DRQ10

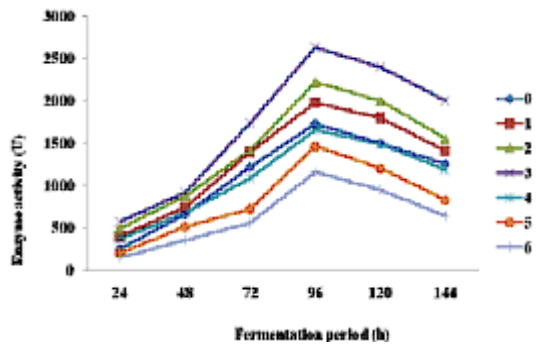


**Fig. 5.** Influence of sodium chloride concentration on the production of amylase by the novel actinobacterium DRQ10

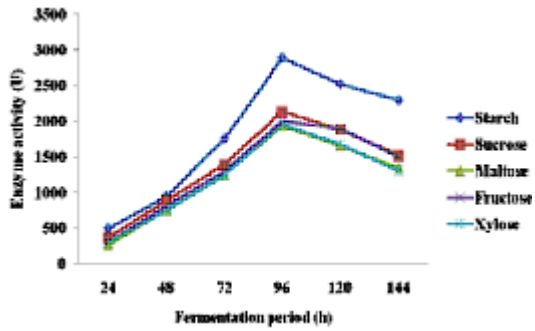


**Fig. 6.** Influence of carbon source on the production of amylase by the novel actinobacterium DRQ10

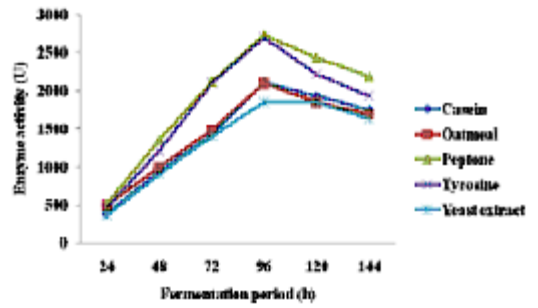


**Fig. 7.** Influence of nitrogen source on the production of amylase by the novel actinobacterium DRQ10

was aimed to isolate amylolytic actinobacteria from the limestone quarries of Deccan traps, from Gulbarga and Betamcherla. The soil scrap of limestone quarries contains a diverse community of organisms differentiated by morphology and biochemical characters. The morphological characters of the selected actinobacterial isolates were shown in Table 1. The enzymatic hydrolysis of starch has been practiced on an industrial scale for many years and is gradually replacing the traditional acid hydrolysis process[25]. To study the production of α-amylase, the isolates of actinobacteria were inoculated into a suitable medium and morphological characters were studied. The isolates were screened for amylase production on starch agar plates with iodine solution. Amylase producers were confirmed by observing clear zone around the colony (Fig. 1).

High biomass of amylolytic actinobacteria was observed after 96 hour fermentation period for all the potential isolates. The isolate DRQ10 showed highest biomass after 96 hours than the other isolates. The actinobacteria isolates synthesized amylase at pH 9.0, the production decreases slowly beyond pH 9.0, this indicates that the enzyme is optimally produced under alkaline conditions. Alkaline amylases are thought to be important from industrial point of view. Production of amylase was maximum at temperature 45 °C, indicating the thermotolerant nature of the organism. The thermostability of the enzyme can be favourable in industrial operations for traditional brewing and food processing, where temperatures of pasteurization could be used to inactivate the enzymes after fermentation (Stamford et al., 2001). Thermostable amylase has been reported to be active at 57 °C by *Pencillium rugulosum*[26]. A thermophilic spore-forming strain was isolated from geothermal soil in Antarctica was thermotolerant, and grew well between 45 and 65 °C (optimum at 61 °C) [27]. *Bacillus subtilis* strain DMO3 grown optimally at 52–55 °C and secret significant amount of alpha-amylase at pH 8[28]. Amylase production was maximum at 3 M NaCl concentration than in absence of salt, and the production declined with further increase in the salt concentration. Tolerance to high salt concentration designates the halophilic nature of the isolates. Organisms with thermophilic, alkaliphilic and halophilic properties can be explained by the calcite rock habitat of the isolates and all the isolates showed similar properties. Amylases with thermophilic, alkalophilic and halophlic characteristics have wide range of industrial applications.

The effect of different carbon and nitrogen sources on amylase production was studied by their addition to nutrient broth. Although the isolates utilized starch, sucrose, maltose, fructose and xylose, starch (0.5%) and sucrose (0.5%) gave significantly higher yield of amylase than other carbon sources added to nutrient broth. Peptone and tyrosine was the most suitable for amylase production than the other nitrogen sources used. Carbon and nitrogen sources are essential for the growth of isolates and optimum production of amylase. The requirement may be explained by the organism's preference for the carbon and nitrogen source for growth rather than for extracellular enzyme formation.

## CONCLUSIONS

This study clearly shows that the actinobacteria isolated from the limestone quarries of Deccan Traps possess high potential for producing amylases of thermophilic, alkaliphilic and halophilic nature. Among the sixty three isolates, six isolates were found to possess amylase activity and DRQ10 containing maximum activity.

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