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# Thermo and Alkali Tolerant Exo-Inulinase Produced by *Streptomyces* sp. Isolated from Unexplored Terrestrial Habitat

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**KEYWORDS**

Citadels, Actinobacteria, Exo-inulinase, Streptomyces, Unexplored habitat, 16S rRNA, Phylogenetic tree

**A B S T R A C T**

Citadels are the old construction sites on top of the hills from an ancient history. During these constructions, there was no practice in use of cement which is replaced by mortar (masonry) composed with calcium carbonate, sugar, stony sand, water. As these site are deteriorated with microbial actions on degrading the walls and paints, our intention made us to isolate the different actinobacteria from this unexplored habitat. In our present study, 48 isolates were obtained from Bidar and Koppal citadels which were further subjected for the screening of Exo-inulinase enzymes (which has less reported from *Streptomyces* sp.). Among 48, 10 isolates were positive and among which KF-5 showed the highest zone of hydrolysis (80mm) ever reported. Using KF-5, flask level fermentation with optimized temperature at 63$^{o}$C and pH at 10 was performed and estimated the concentration of enzyme produced about 63µg/ml by FCR (Folin–Ciocalteu reagent) method. The species identification was done by 16S rRNA sequencing resulted as hitting to *Streptomyces albus* with 94%, further subjected for poly-phasic analysis to prove its novelty.

## Introduction

Citadels are some of the oldest known structures which have served as boundaries of the kingdom territories and centralised authority which were built by the Indus Valley Civilization [1]. The wall of these citadels are built with help of mortar, a composition of calcium carbonate, sugar, stony sand and water, which is used as strong binder between big rocks [2]. Deterioration is the major bout for the citadels as the microbial interaction with these surfaces destroys the texture of the

mortar. Thus, many microbial interactions might involve in this process, since the present investigation tuned up for the isolation of actinobacteria from 2 districts of Hyderabad-Karnataka region such as Bidar and Koppar.

*Actinobacteria* are the class of microbes which resembles fungi, but their characteristic had major differences between them. 80% of the antibiotics are reported from *actinomycetes* itself, among these 40-45% percent belongs to *Streptomyces*. Comparatively enzymes from *Streptomyces* are reported very less, exo-inulinase is one among them.

There is an increasing demand for a safe alternative sweetener to sucrose in order to mitigate the health issues caused by it. Fructose and its oligosaccharides are one such choice of sweeteners which over take sucrose in terms of higher solubility, lower viscocity, low calorie diet and *Bifidus* stimulation [3, 4, 5]. Oflate, microbial inulinases have grabbed an attention as an industrially important class of enzymes that hydrolyze inulin to produce fructose and fructooligosaccharides. Inulin is a polyfructoside reserve that occurs in tubers of Jerusalem artichoke, Chicory, Dahlia, Dandelion and Burdock [6]. Being abundant in nature, inulin serves as an inexpensive substrate for fructose production in various food and pharmaceutical industries.

Inulin is as natural polysaccharide found in many plants as reserved material, excessively present in garlic. The fructosyl units in inulin are attach with $\beta$ (2 -1) linkages with terminating glucose residues. Hydrolysis of inulin produces fructo-oligosaccharides and fructose, which promising molecules of food and pharmaceutical and energy industries [7]. Exo-inulinases (EC 3.2.1.80) are most efficient in hydrolyzing the inulin for fructose which yields as high as 90-95% [8]. Thus microbial exo-inulinase has much demand in production fructose syrups which is most important and high sweetner used in food as preparations of jellies, candies, chocolates etc. and in pharmaceutical as preparation of medical syrups, tablets, etc. This great demand over tuned to start with the higher microorganism such as *Streptomyces* sp. In the present study, synthetic inulin is used for screening of exo-inulinase enzyme by *Streptomyces* sp. isolated from new area of interest and unexplored till date.

## Materials and Method

### Isolation of Potent Actinomycetes from Citadels

Soil samples from unexplored citadels of Bidar and Koppal districts of Karnataka, India, were collected and the colour (Fig. 1), pH and temperature of the soils were checked before they were being exploited for isolation of actinobacteria. All the soil samples were separately mixed with calcium carbonate and kept at room temperature for successive 5 days, in order to kill the bacillus, other bacteria and enrich the actinobacterial spores. After treatment, the samples were serially diluted [9] and spread plated on to Starch Casein Agar (SCA) for the isolation of actinobacteria. Constituents of SCA (g/L): Soluble Starch-10; $K_2HPO_4$-2; $KNO_3$-2; NaCl-2; Casein-0.3; $MgSO_4.7H_2O$-0.05; $CaCO_3$-0.02; $FeSO_4.7H_2O$ and Agar 15.

### Screening of Exo-inulinases Producing Actinomycetes

Based on the colony morphology and number of colonies, 48 isolates were subcultured on SCA by quadrant streak method. The selected strains of

actinobacteria were used for screening of exo-inulinase producer on 150mm petridishes containing Czapex Dox Agar (CDA) medium comprising the following constituents (g/L): $NaNO_3$, 3.0; $K_2HPO_4$,1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; inulin 10 and agar 15, pH 8.5 [10]. All isolates were spot cultured (as a size of antibiotic disc) on CDA and incubated at 37$^oC$ for 3 days.

After successive incubation period, the isolates doesn't show the zone hydrolysis until the plates treated with detection reagents, such as TTC reagent (0.1% triphenyl tetrazolium chloride in 0.5M NaOH) for 20 mins in the dark [11]. After washing in the 0.1 M acetate buffer (pH 5), the extracellular production of inulinase was confirmed by red zone of hydrolysis around the colonies (Fig. 2).

## Morphological and Biochemical Characterization of Potent Exo-inulinase Producers

Positive isolates from the screening were subsequently studied for morphological and biochemical characterizations such as IMViC, Catalase, Gelatin Liquefaction, Triple Sugar Iron. Morphological examinations like Grams staining and Spore staining by microscopic observations.

## Effect of Temperature and pH on Growth and Exo-inulinase Production

Prior to start the production of exo-inulinases through flask level fermentation, temperature and pH optimization was performed to check optimum conditions required for the isolate to produce the enzyme in excess. Different temperatures were adjust such as 7$^oC$ to 63$^oC$, with the difference of increasing temperature as 7$^oC$. For pH optimization, the flasks were adjusted with 1 to 14 pH, inoculated with test positive isolate and incubated at 60$^oC$ for 3 days. The results of both growth and inulinase activity were analyzed spectrometrically at 660nm, values are constructed with graph.

## Flask Level Fermentation for Exo-inulinase Production

Shake flask fermentation is small scale fermentation which is most optimistic for research purpose. With the tested optimized conditions, in a clean 1000ml Erlenmeyer flask, CD (Czapex Dox) broth was prepared with 1% inulin as substrate, then inoculated with 2ml of 24hrs culture broth of KF-5 and incubated at 60$^oC$ for 72 hrs., for the production of exo-inulinase enzyme.

## Inulinase Assay

Crude enzyme was retrieved by centrifugation (10000 rpm, 4$^oC$), and diluted in 2M Urea buffer and stored at 4$^oC$ until use. One unit of inulinase activity will be determined based on the quantity of fructose liberating (1µmol/min) into the medium. For 1ml of 0.5% (w/v) inulin, 0.2ml of crude extract supernatant was added and incubated at 60$^oC$ for 15mins. Later the amount of fructose released was accessed by FCR methods and Nelson-Somogyi method and observed OD at 660nm.

## Agar Well Diffusion Assay with Crude Enzyme Extract

The crude enzyme extract was used for well diffusion assay to analyze the enzyme presence in the supernatant. The rate of Diffusion and hydrolyzing inulin present in the solid state will be compared between screening plates and diffusion plates. CDA plates were bored with well of 5mm width, about 50-100µl of crude enzyme was loaded and incubated at 37$^oC$ for 24 hrs.

## Results and Discussion

### Isolation of actinobacteria from soil samples of Citadels.

The collected soil samples from Bidar and Koppal district, Karnataka, India, were subjected for pH determination, all samples were within the range of 8.0-8.5±0.2. The temperature of the soil at the collection sites was ranging from 30°C to 42°C. After successive isolation, 48 isolates were obtained from the processed soil samples. These isolates were subjected for further screening of enzymes.

### Screening of Exo-inulinases

All obtained 48 isolates were subjected for screening of exo-inulinases on CDA medium (Fig. 2), among them 10 isolates showed positive and zone of hydrolysis measured as shown in Table 1, of which KF-5 isolate expressed much zone of hydrolysis of about 80mm in diameter (Fig. 3). All 10 isolates were sub-cultured on SCA and preserved for further use.

### Morphological and Biochemical Characterization

Microscopically all 10 isolates are gram positive, spore bearers and non-motile, KF-5 isolate is gram positive and spore bearer (Fig. 4). Biochemical tests performed for the isolates and observations are tabulated in Table 2. Among them KF-5 and KF-8 has shown similar, KF-5 showed positive for Voges Proskeur, Catalase, Triple sugar iron tests.

### Effect of Temperature and pH

Every microbe express some different features when their growth conditions altered. Temperature optimization study indicates that KF-5 sustains the temperature 60°C both for growth and enzyme production (Graph 1). pH optimization study of KF-5 revealed that, it will sustains the highest pH up to 10, for both growth and enzyme production (Graph 2).

### Flask Level Fermentation

Fermentation for the production of exo-inulinase was done in 1000ml Erlenmeyer's flask, with the optimized conditions. During fermentation, in successive intervals the media was checked for the turbidity and colour. As KF-5 is a mild melanin producer, the production broth was turned to light brownish colour. The temperature and pH of the medium was subsequently checked every day during fermentation. After completing the incubation time broth was drawn for further quantitative analysis.

### Inulinase Assay

The amount of reducing sugar in the production broth determines the units of enzyme produced to hydrolyse the substrate. The reducing sugar fructose was determined by FCR method, using BSA as standard. The concentration of enzyme estimated is ~6.3mg/ml (Graph 3) and it is expressed as 0.894 IU/ml, against the standard BSA curve. In turn if 1ml gives concentration of 63µg, for 300ml approximate volume of enzyme is 18.9g, which is a good concentration and highest ever reported.

### Well Diffusion Assay for Crude Enzyme Extract

Confirmation of enzyme in the production medium was determined by well diffusion assay. Zone of hydrolysis shown for 100µl of crude enzyme extract is 63mm in diameter (Fig. 5). Difference between the screened plates (with culture) and diffusion plate could be well determined, because of presence of culture in the screened plates

will continuously secrets the enzyme, but in the crude extract, a definite concentration of enzyme showing pale pink colour.

**Table.1** Zone of Hydrolysis of Potent Inulinase Producing Isolates

| Sl.no | Name of isolate | Zone of hydrolysis (in mm) |
|---|---|---|
| 1 | BiF23 | 24 |
| 2 | KF2 | 40 |
| 3 | KF3 | 30 |
| 4 | KF4 | 35 |
| **5** | **KF5** | **80** |
| 6 | KF8 | 45 |
| 7 | KF9 | 36 |
| 8 | KF13 | 40 |
| 9 | KF15 | 40 |
| 10 | KF16 | 24 |
| 11 | KF19 | 30 |

**Figure.1** Texture and Colour of Different Soil sSamples Collected from A) Bidar and B) Koppal

**Figure.2** Plates Showing 47 Isolates Screened for Exo-inulinases

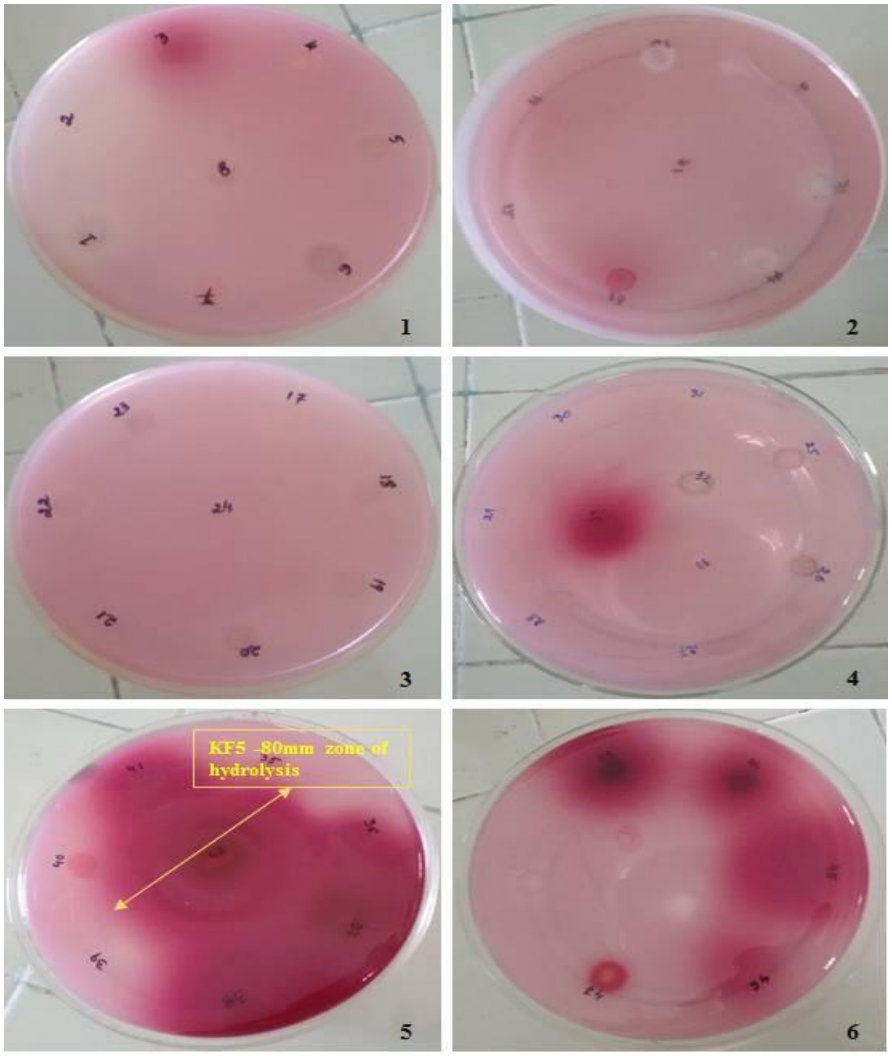



**Figure.3** Single Plate of KF5 Isolate Showing Zone of Hydrolysis for Exo-inulinase Activity

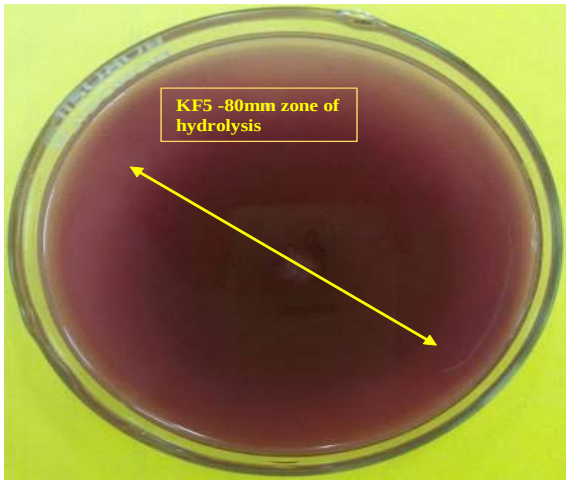


**Figure.4** Gram's Staining of KF5 Isolate

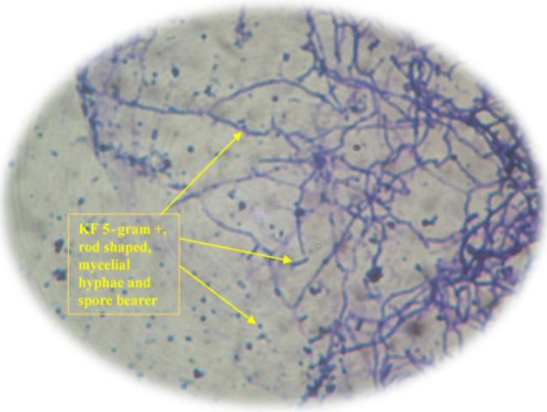



**Figure.5** Well Diffusion Assay for Crude Enzyme Extract

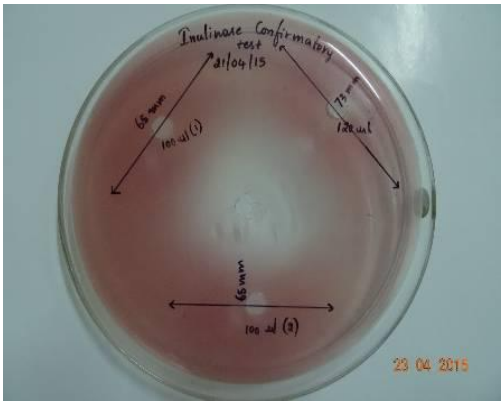
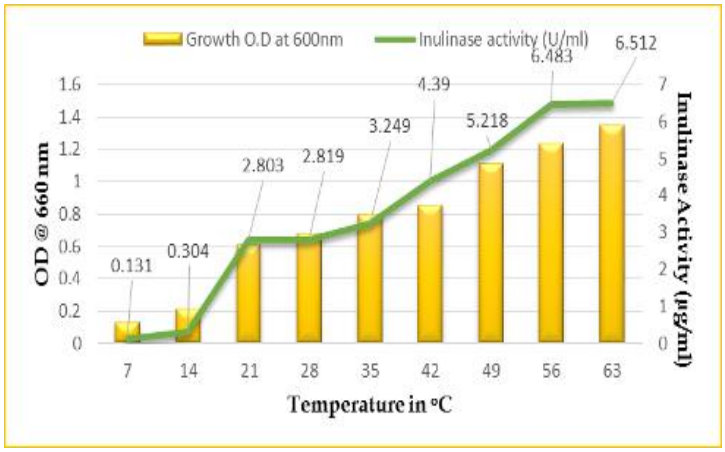


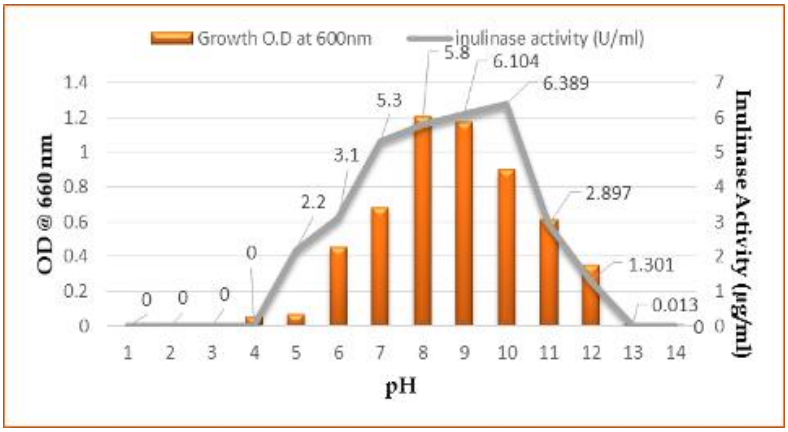

**Table.2** Biochemical Characteristics of 10 Positive Isolates which Secretes Inulinases

| TEST | Results | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | BiF23 | KF2 | KF3 | KF4 | KF5 | KF8 | KF9 | KF13 | KF15 | KF19 |
| Methyl red | + | + | + | + | - | - | + | + | + | - |
| Voges Proskeur | - | - | - | - | + | + | - | - | - | + |
| Indole | - | - | - | - | - | - | - | - | - | - |
| Citrate utilization | + | - | - | - | - | - | - | - | + | - |
| Catalase | + | - | - | - | + | + | - | - | + | - |
| Gelatin liquefaction | + | + | + | + | - | - | + | + | + | - |
| Triple Sugar Iron | + | + | + | + | + | + | + | + | + | + |

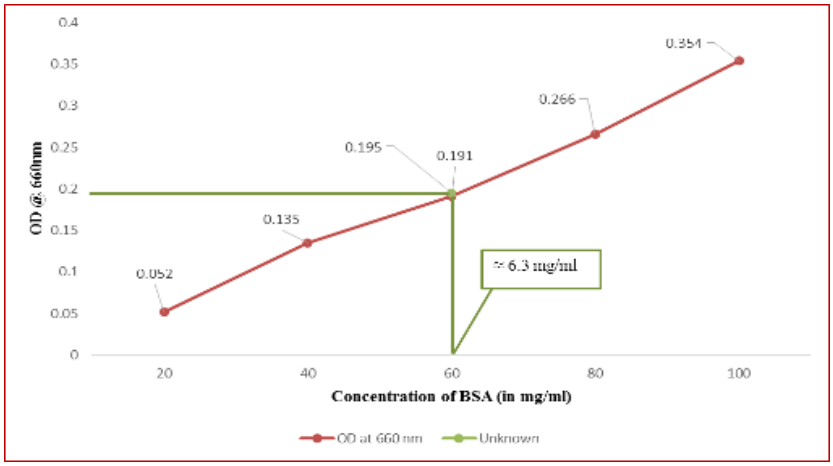
**Graph.1** Effect of Temperature on Growth and Enzyme Activity of KF5




**Graph.2** Effect of pH on Growth and Enzyme Activity of KF5




**Graph.3** Estimation of Exo-inulinases from Production Broth by FCR Method



Industrial exploitation of actinobacteria from different unexplored habitats is the major task that adds the novel microbes into the pockets of emerging industries. The production rate of exo-inulinase if high as compare with the other organisms *Bacillus* sp. [12], *A. niger* A42 [13] and *A. fumigatus* [14]. The isolates from this habitat are still under identification process with 16S rRNA, Polyphasic analysis. Also the purification and characterization of exo-inulinase isolated are under progress. The other optimization studies like effect of metal ions, substrate concentration, different natural substrate are conducted which is under communication for publication.

The concentration of the enzyme reported from other organisms such as 0.230 IU/ml from *Panaeolus papillonaceous* [15], 0.222 IU/ml from *Candida kefyr* [16], 0.392 IU/ml from *Kluyveromyces fragilis* [17] and 0.552 IU/ml by Streptomyces sp. GNDU 1 [10]. Comparatively the high thermo tolerant reported from other organisms such as *Streptomyces rochei* E87 at 32$^{o}$C [18], *Aspergillus niger* at 28$^{o}$C [19], *Penicillium janczewski* at 28$^{o}$C [20] and 46$^{o}$C by Streptomyces sp. GNDU 1 [10].

Over all compare to other microbial exo-inulinase our newly isolated *Streptomyces* sp. exo-inulinases are much potent in terms of temperature, pH and production rate. The isolate KF-5 is still needed to prove novelty by other characterization studies.

In conclusion, many terrestrial environments are still has to dig up with novel microorganisms such as quarries, citadels, cement factories and other artificially man made environments. A novel organism can have dramatic genome hidden with potent gene clusters, they will express only if they are provided with specific substrates. The exo-inulinase isolated in this study is a promising molecule for industrial exploitation, which is under progress for the large scale development.

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