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# Subterranean Depth Dependent the Modulation of Endo and Exoenzyme Secretion in *Streptomyces prasinosporus:* A Common Soil Actinomycete of India

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**Actinomycetes are the most accustomed biotic component of almost all the subterranean caves of the world. The smell of this particular microorganism is indeed a typical characteristic of all the caves. Moreover, the *Streptomyces* sps. are also found to be highly useful for its highly potent enzyme producing properties, useful for various biotechnological tools and also medically useful antibiotic properties. In the present study the molecular confirmation of a particular actinomycete species *Streptomyces prasinosporus* occurring at various habitats of Kotumsar cave has been confirmed. Further the endo and exoenzyme secretion capacities of two important enzymes of the same actinomycete have been compared with respect to its occurrences in various cave habitats.**

**Key words**: Kotumsar Cave; *Streptomyces prasinosporus;* 16S rDNA; Alkaline protease, Glucose isomerase.

Due to various unusual conditions existing among biotic and abiotic factors inside the subterranean caves, it usually represents an extreme fragile environment in the earth. The complete lack of light inhabits the autotrophs which ultimately designates the cave as energy starved biome and perhaps simultaneously encourages the competition among its microbial community for successful survival. Thus, the microbes isolated from such hostile biospheres are proven comparatively to be more potent, how far our biotechnological tools are concerneded (Cabeza *et al*. 2011; Cavicchioli *et al*. 2011; Sanchez *et al*. 2009; Van den Burg 2003). It is well established that the typical environmental conditions of the cave usually favour the growth of actinomycetes / actinobacteria (Groth & Saiz-Jimenez 1999; Groth *et al*. 2001; Rajput *et al*. 2012a) due to which the study of cave microbes is always interesting for discovering potential enzymes.

Till date universally, varieties of bacteria belonging to the actinobacteria (actinomycetes), cyanobacteria, and even archaebacteria have been reported from the subterranean caves (Barton & Northup 2007; Tomova *et al*. 2013; Rajput *et al*. 2012a,b). Among them the genus *Streptomyces* of actinomycetes is the most puissant group of

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microbes which secretes some bioactive compounds to degrade relatively complex and refractory plant and animal residues. Indeed the occurrences of such bioactive compounds in *Streptomycetes sps.*, are the only compound uses in antibiotics and other useful enzymes for us.

In the present era the molecular systematic methods based on 16S rRNA gene sequence and DNA:DNA associated data to establish the taxonomic relationships are getting much importance especially on *Streptomycete* systematic (Kim *et al*. 1996; Rosselló-Mora & Amann 2001; Girard *et al*. 2013). The Amplified Ribosomal DNA Restriction Analysis (ARDRA) technique is one of the major tools to verify microbial diversity which is solely based on DNA polymorphism (Deng *et al*. 2008; Tomova *et al*. 2013). In addition, 16S rRNA sequence data have proved invaluable in *Streptomycetes* systematic, in which they have been used to identify several newly isolated *Streptomyces* species (Bieble & Sproer 2002; Geng *et al.* 2008).

Following the morphological, physiological and biochemical characterizations, previously we have already reported the existence of few actinomycetes in the sediments of Kotumsar cave. Among them *Streptomyces prasinosporus* are the most common isolated from three different habitats of Kotumsar cave; entrance zone (KCA3), transient zone (KCA8) and deep zone (KCA22) (Rajput *et al*. 2012a). Though the phenotypic identification of bacteria carries a high risk of misidentification, in the present study we compared the results of biochemical identification of the same cultures of actinomycetes with 16S rDNA analysis. In addition to the above cultures few more cultures were also considered; *S.luridus* (KCA13), *S.roseus* (KCA18), *S.longisporoflavus* (KCA23) to confirm the species in molecular level.

*Streptomyces prasinosporus* is comparatively a new species (Tresner *et al.* 1966) commonly found in every type of soils in India. Till date very limited attempt has been taken to understand its various biological characteristics. By keeping the same in the mind in the present study we have estimated and compared the extra and intracellular Alkaline protease and Glucose isomerase secretions of all the three cultures of *S.prasinosporus* isolated from three different habitats of Kotumsar cave.

## MATERIALS AND METHODS

### Study site

Kotumsar cave complex is composed of Kanger limestone of Indrawati group of rock of Upper Proterozoic. It is situated in the Kanger Valley National Park (18°50'-61 18°53'48"N, 81°55'-81°58'24"E; 42 $Km^2$; S.O.I. Toposheet No.65F/13) at an altitude of 560 m in Jagdalpur, Baster, India. A permanent stream of River Kanger is always flowing at the foothill where the cave is situated. The main entrance of this cave is formed by a vertical fissure in the wall of a hill, which further leads inwards via a narrow, twisted tubular path, measuring about 15 m in length. The complete cave is nearly 150 m long at a stretch which having several lateral and downward passages leading to several irregular chambers (Biswas 1992a). The ambient external surface of this cave is covered by deciduous to mixed forest vegetation. The air and water temperatures of the cave remain relatively stable at an annual average of 28.25±1.23°C and 26.33±0.96°C respectively (range = 25.0–32.7°C for air; 22.9–29.3°C for water). The water pools were reported to be distinctly Alkaline with an annual average pH value of 8.04±0.36. The Kotumsar cave is subjected to tremendous flooding specially during rains which starts from June and continuous till the end of September in Kanger Valley National Park. The annual mean of percentage saturation for oxygen in this cave water were seen to 74.83 ± 5.91% (Biswas 1992b).

The sediment samples for isolations of *Streptomyces sps.* were collected from the entrance zone (nearer to the entrance of cave), transient zone; just few meters after the crossing of twisted vertical path and the deep zone i.e., around 70 m deep inside the cave (Fig.-1 a,b).

### Species Confirmations

In the present study we used earlier isolated cultures, identified on the basis of morphological, physiological and biochemical characterizations by using PIBWin programme. *PIBWin* programme is based on a system of probabilistic identification of unknown *bacterial* isolates against identification matrices of known strains (Bryant, 2004). As stated earlier, three cultures of *Streptomyces prasinosporus* and three others, *S. luridus, S.roseus, S.longisporoflavus* were chosen from the stock cultures as the source

of template DNA for ARDRA analysis.

Further, for Amplified Ribosomal DNA Restriction Analysis (ARDRA), the isolation of DNA was extracted by snap-chill method (Winship 1989). *Streptomyces* isolates were grown in 10 ml LB broth for 18–24 h and were harvested by centrifugation (7500 g for 2 min), washed once with 500 µl of TE buffer (pH 7) and resuspended in 500 µl TE buffer (pH 7). The samples were heated in boiling water for 10 min. Then suspension was snap chilled at -20°C for 5 min and centrifuged (7500 g for 3 min). The supernatant (300 µl) was transferred to a clean tube and stored at 4°C. This supernatant containing DNA was used as template for PCR amplification. 16S rRNA gene amplification, reaction mixture contained the following reagents: 2.5µl of 10X reaction buffer [500 mM KCl, 100mM Tris-Cl (pH 8.3)], 2.5µl of a solution containing each of the deoxynucleoside triphosphates at concentration of 2.5mM, 2.5 µl of 25mM $MgCl_2$, 20pico mol of primer, 1U of r Taq DNA polymerase and 3.0µl of template F-5'AGAGTTTGATC CTGGCTCAG3' (pA) and R-5'AAGGAGGTGAT CCAGCCGCA3'(pH9), oligonucleotide was desalted oligo (yield: 2-3 O.D) (Thorn and Tsuneda, 1996). The reaction volume was adjusted to 25µl using sterile double distilled water. The reaction mixtures were subjected to initial denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min., 36°C for 1 min. and 72°C for 2 min., and a final extension step at 72°C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel and visualized under an UV transilluminator. Each 20µl reaction contained 10 µl of PCR product, 1.5 µl of *Hae*III enzyme (1U), 1.5 µl buffer (1X), 7 µl double distilled water and kept in water bath at 37°C for 12 h. The reaction product was analyzed by electrophoresis on a 2% agarose gel, staining with ethidium bromide and gels were photographed using a digital camera system (Nikon, Coolp1X995, Japan), and the patterns were compared visually.

ARDRA bands were scored as either present (1) or absent (0). All binary data were entered and genetic distances were calculated through Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), version 2.02 and calculating Euclidean distance and then assembling a dendrogram using "Unweighted Paired Group Method using Arithmetic average criterion" (UPGMA).

**Screening for energy providing enzymes**

The endo and exozyme secretions of both the enzymes, *i.e.*, Alkaline protease and Glucose isomerase were estimated in all the three cultures of *Streptomyces prasinosporus*. Quantitative estimation of Xylose (Glucose) isomerase was done by cystine/ carbazole/ sulphuric acid method (Dische & Borenfreund 1951). Alkaline protease activity was determined by the method forwarded by Nakagawa (1970).

**Statistical analysis**

Two-way ANOVA was employed to compare the endo Vs exoenzymes secretions of both Alkaline protease and Glucose isomerase separately in *Streptomyces prasinosporus*, with respect to their various habitats of isolations inside the Kotumsar cave. Further, student's "t" test was also employed separately to compare the endo Vs exoenzymes secretions separately in each case.

## RESULTS

Results are summarized in Table 1 – 2a,b and Figure 2 to 4a,b**.** In the present study, ARDRA pattern of *Streptomyces sps.* were analyzed by using universal primer and endonuclease enzyme *Hae III.* The dendogram generated on the basis ARDRA pattern using *HaeIII* showed that three isolates of *Streptomyces prasinosporus*, each from entrance zone, twilight/transient zone and deep zone revealed complete similarity, whereas *Streptomyces roseus* KCA 13 from transient zone, *Streptomyces longisporoflavus* KCA18 and *Streptomyces luridus* KCA23 were obtained in different clades. The results presented here also indicate that *HaeIII* the restriction enzyme was successful in characterizing the strains belonging to the same genera (Table-1, Fig. 2-3).

Further, while comparing the endo and exoenzyme Alkaline protease secretions, we found endoenzyme secretions were always copious than its respective exoenzyme counterparts. While applying the two way ANOVA, a highly significant cellular effects (ecdo Vs exo) on the secretions of Alkaline protease ($p<0.001$) was evident. Further, the respective distances effect on the level of secretions was also witnessed ($p<0.001$) i.e., longer the distances between the cave entrances and the respective habitats of culture isolation lesser the secretion of this particular enzyme was recorded.

**Table 1.** Confirmations of the *Streptomyces sps.* based on 16SrDNA fragmentations' results

| S. No. | Zone | Culture Code | *Streptomyces sps.* | *Hae III* digested 16S rDNA fragment ( bp) |
|---|---|---|---|---|
| 1. | Entrance zone | KCA3 | *S. prasinosporus* | 700, 600, 590 |
| 2. | Transient zone | KCA8 | *S. prasinosporus* | 700, 600, 590 |
| 3. | Transient zone | KCA13 | *S. roseus* | 620, 580, 560, 550 |
| 4. | Deep zone | KCA18 | *S. longisporoflavus* | 590, 560, 550 |
| 5. | Deep zone | KCA22 | *S. prasinosporus* | 700, 600, 590 |
| 6. | Deep zone | KCA23 | *S. luridus* | 620, 600, 590 |

**Table 2(a).** ANOVA Summary of Alkaline protease

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Habitats | 8463.5 | 2 | 4231.75 | 84.81 | <.0001 |
| Cellular | 1154.72 | 1 | 1154.72 | 23.14 | 0.0004 |
| H x C | 925.07 | 2 | 462.54 | 9.27 | 0.0037 |
| Error | 598.75 | 12 | 49.9 | | |
| Total | 11142.04 | 17 | | | |

**Table 2(b).** ANOVA Summary of Glucose isomerase

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| Habitats | 539.19 | 2 | 269.6 | 1050.37 | <.0001 |
| Cellular | 65.23 | 1 | 65.23 | 254.14 | <.0001 |
| H X C | 124.7 | 2 | 62.35 | 242.92 | <.0001 |
| Error | 3.08 | 12 | 0.26 | | |
| Total | 732.2 | 17 | | | |

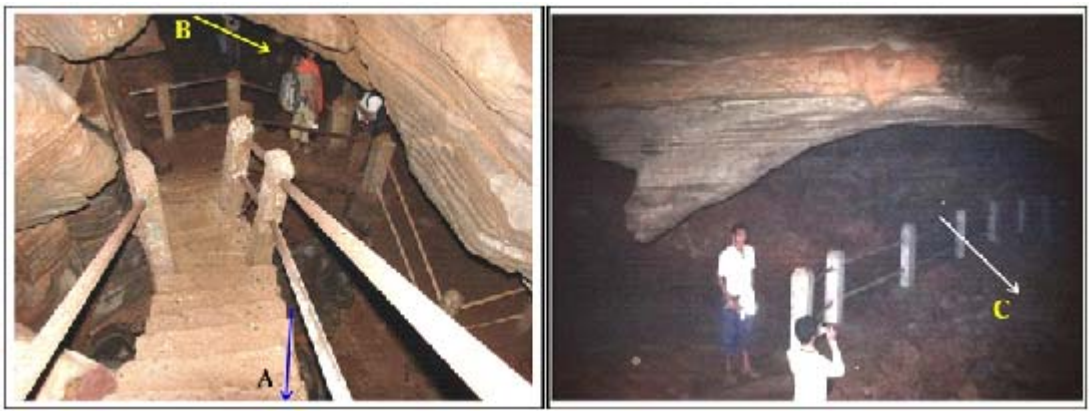


**Fig. 1.** Arrow indicating the spots from where the sediment samples were collected for isolation of *Sptreptomyces sps.;* A- Entrance zone, B- Transient zone, C- Deep zone (inner view of the cave). Typical Kanger Limestone patterns are apparent in sidewalls of both the figures

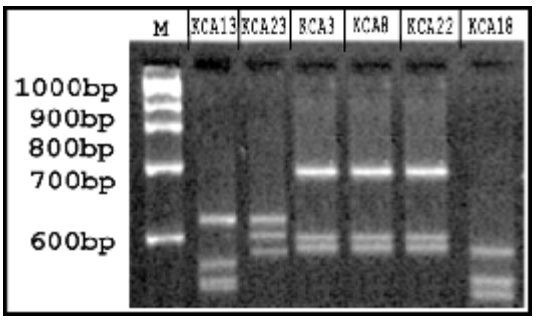


**Fig. 2.** ARDRA pattern using *HaeIII* from Streptomyces *sps.* 253 with universal primer

The interaction effects of above two was also found to be highly significant ($p<0.004$).

More or less the same ANOVA results were evidenced in the case of enzyme; Glucose isomerase secretion. All the effects i.e., cellular, distance and interaction were found to be highly significant ($P< 0.001$). However, here the exoenzyme secretions were found to be more as compared to its endoenzyme counterparts. Interestingly the cultures isolated from the transient zone secreted both the ecdo and exoenzyme Glucose isomerase in almost same proportion.

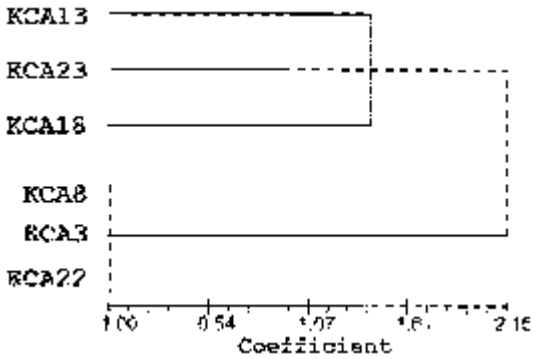


**Fig. 3.** Dendogram showing relatedness among *Streptomyces sps.* based on restriction digestion of 16SrDNA using *HaeIII.*Whereas, KCA13: *Streptomyces roseus* KCA13; KCA23: *Streptomyces luridus* KCA23; KCA3: *Streptomyces prasinosporus* KCA3; KCA8: *Streptomyces prasinosporus* KCA8; KCA22: *Streptomyces prasinosporus* KCA22; KCA18: *Streptomyces longisporoflavus* KCA 23

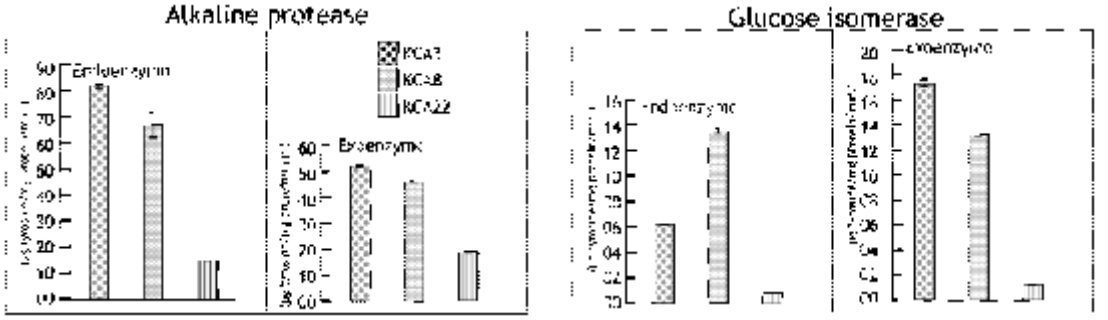


**Fig. 4(a).** Comparative bar diagrams representing the level of secretions of Alkaline protease and Glucose isomerase among three different cultures of *Streptomyces prasinosporus* isolated from three different habitats of Kotumsar cave; KCA3- Entrance zone; KCA8- Transient zone; KCA22- Deep zone. In all the respective groups, each bar is statistically differing from the other at less than 01% level

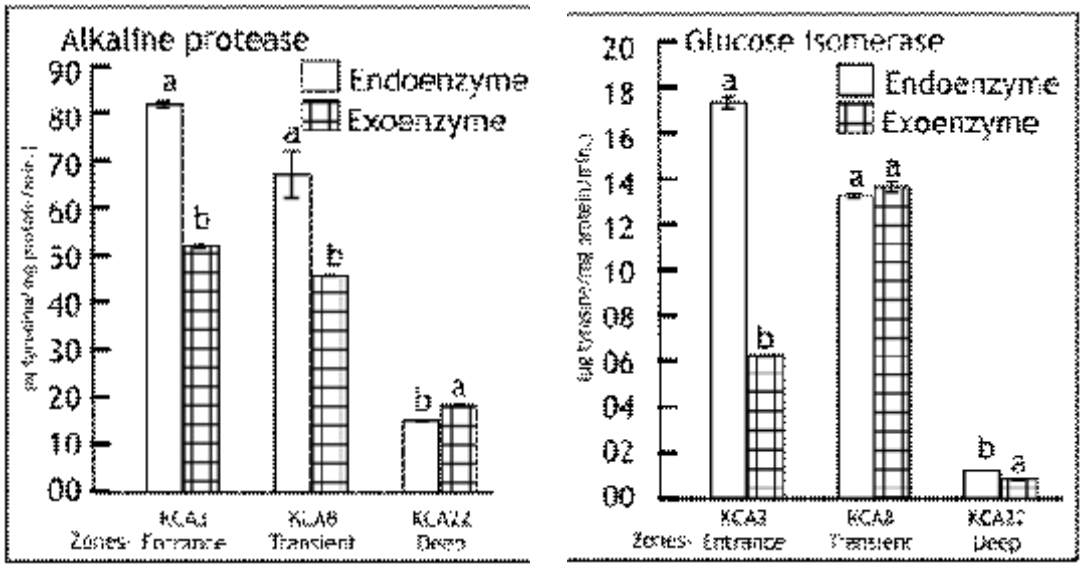


**Fig. 4(b).** Comparative bar diagrams representing the level of respective Endo and Exoenzyme secretions of Alkaline protease and Glucose isomerase separately in three different cultures of *Streptomyces prasinosporus* isolated from three different habitats of Kotumsar cave; KCA3- Entrance zone; KCA8- Transient zone; KCA22- Deep zone. Similar superscripts do not differ from each other at even 5% level

The overall level of secretions of both the enzymes; Alkaline protease and Glucose isomerase were found to be always high in the cultures of *Streptomyces prasinosporus* isolated from the entrance zone then transient and deep zones (Fig.-4a). Interestingly the secretions of endo enzymes lowers significantly down than its exoenzyme counterparts in both the cases which were isolated from the deep zone (Fig. -4b).

## DISCUSSION

In any subterranean cave, the scope for occurrences of varieties of actinomycetes is always high. Indeed the characteristic smell coming out from most of the caves are mainly due to the production of an original metabolite (geosmine) of actinomycetes (Moore & Sullivan 1997). Some of the species have also been reported to participate in the moonmilk production inside the cave (Cañavaras *et al*. 1999; Groth & Saiz-Jimenez, 1999; Boston *et al*., 2001; Braissant *et al*., 2012; Baskar *et al*., 2011). The extracellular organic compounds produced by these microorganisms are very helpful in minimizing the gap between the biological processes with its surrounding geo-chemical ones (Stone 1997).

It is well established that the bacterium usually do not secrete the enzymes to metabolize such substrates which do not exist in its growth medium. Thus, some enzymes are not synthesized in certain cells, whereas other are synthesized only as per the need, as exception few are found in all cells. In this aspect the exoenzyme secretion plays a key role in degradation and utilization of organic polymers existing around it, though only molecules having mass lower than 600 daltons can pass through the respective cell walls (Vasileva-Tonkova & Galabova, 2003).

*Streptomyces prasinosporus* is comparatively a new member of actinomycetes group occuring in every types of soil in India (Tresner *et al*., 1966). In the present study both the endo and exoenzyme, Alkaline protease secretions were found to be statistically very high in the cultures which have been isolated from the entrance than the transient zones. The lowest content of both the enzymes were always evident from the cultures which were isolated from the interior (deep) zones. Further, the endoenzyme, Alkaline protease secretion in *Streptomyces prasinosporus* was always found to be statistically very high while compared with their respective exoenzyme counterparts. It was signally in those cultures which have been isolated from the entrance than the transient zones. It clearly interprets that the deep zone of the cave lacks such organic component/media (especially protein) the degradation of which is Alkaline protease dependent or in other words the protein content of the ambient media of *S. prasinosporus* gradually decreases from entrance to deep zone.

Surprisingly, while comparing endo Vs exo enzyme Glucose isomerase secretions, the outcome was found to be altered upto far extent than the above case (Alkaline protease). In nutshell, the exoenzyme secretion of Glucose isomerase was found to be statistically high as compared to their endoenzyme counterparts' secretion, especially while compared between the secretions of the cultures isolated from entrance versus deep zone. Here it was interesting to note that the endo and exoenzyme secretion of Glucose isomerase remain almost same in the culture, KCA8 which was isolated from the transient zone. In this regard, the endoenzyme secretion of Glucose isomerase was found to be highest in KCA8 culture which was isolated from the transient zone. However, in the present situation, we are here not in a position to interpret the reason.

Conclusively, the secretions of both the enzymes; Alkaline protease and Glucose isomerase were found to be usually high in the cultures isolated from the entrance zone which gradually declines as per their occurrences habitat shifted from outer to the deeper zones of the caves. Here it could be safely hypothesized that the organic content of deep zones of Kotumsar cave is too low due to which the exoenzyme secretions of above both the above enzymes lowers down in *Streptomyces prasinosporus*.

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