RESEARCH ARTICLE

Subterranean Microhabitat Dependent Intra Versus Extracellular Enzyme Secretion Capabilities of *Deinococcus radiodurans*

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Study Area: Kotumsar Cave, Jagdalpur, India

Coordinates: : 18°52'090" N; 81°56'05" E

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Abstract

Deinococcus radiodurans is one of the most yet discovered extremophilic microbe, the isolation of which from the various habitats of Kotumsar cave is always a matter of enticement to discover its ecological economics. In the present work we studied the intraversion extracellular alkaline protease and glucose isomerase secretion capabilities of *Deinococcus radiodurans*; KCB21, KCB50, KCB93 isolated from three distinct subterranean niches of Kotumsar cave. The selected niches/zones were the entrance zone, transient zone and the deep inner zone from where the soil sediments were collected to isolate the bacterial strains. The results revealed high extracellular alkaline protease activity from the *Deinococcus radiodurans* strain which was isolated from the deeper zones of the cave, whereas no such phenomenon was revealed for glucose isomerase. The possible reason for the obtained results has been discussed.

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Introduction

Extremophilic species are capable to withstand the extreme ambient conditions and are always considered beneficial for a wide range of research and commercial purposes. *Deinococcus radiodurans* is a gram-positive, pigmented, non-spore forming, non-motile, spherical bacterium ranging from 1.5 to 3.5 μm in diameter and grows with a doubling time of ~ 80 min in a rich nutrient medium. It is one of the most extremophilic members from Deinococcaceae family which has extreme radiation resistant capacity due to which it is often referred as polyextremophile (Slade & Radman, 2011). *D. radiodurans* has already been listed as the world's toughest bacterium in "The Guinness Book of World Records" (DeWeerd, 2002). Nevertheless, till date very little attempt has been made to understand its ecological economics. In general it is difficult to isolate microbes like *D. radiodurans* from common soil, excreta, hay sample or domestic sewage but reports are available supporting its isolation from elephant trunks to granite outcrops in Antarctica are available (Daly & Minton, 1996; Makarova *et al.*, 2001).

Subterranean caves always represent an unusual ecosystem due to some of its uncommon geophysical characteristics viz., perpetual darkness, constant temperature, low energy input, relatively high humidity and CO_2 concentration etc. Further, in such isolated subterranean environments gradual alterations in habitat are also apparent which is mainly due to alterations in internal factors viz., guano deposition, fungal colonies, biofilm/foval soil etc. (Camassa, 1997). Thus the microbes which originate in such extreme environment could be safely referred as extremophilic species. In our earlier studies we have already reported the occurrence of *D. radiodurans* from various habitats (Biswas, 2010) of Kotumsar cave (Rajput *et al.*, 2012). The occurrence of *D. radiodurans* in various habitats of the Kotumsar cave which is an almost energy starved ecosystem perhaps increases the possibility of its potential enzyme producing capabilities.

In the present study we have used three strains of *Deinococcus radiodurans* isolated earlier from the three distinct habitats of Kotumsar cave (Rajput *et al.*, 2012) and tried to compare their intra and extracellular activities of two specific commercially important enzymes, alkaline protease and glucose isomerase.

Materials and Methods:

Study area: Kotumsar cave complex is composed of Kanger limestone of Indrawati group of rock of Upper Proterozoic. 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 $28.25 \pm 1.23^\circ\text{C}$ and $26.33 \pm 0.96^\circ\text{C}$ respectively (range = 25.03 – 32.7°C for air; 22.92 – 29.3°C for water). 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 $74.83 \pm 5.91\%$ (Biswas, 1992b). The sediment samples for isolations of *Deinococcus radiodurans* were isolated from the entrance zone 'A' (nearer to the entrance of cave), transient zone 'B' (just few meters after the crossing of twisted vertical path) and the inner deep zone 'C' (around 70 m deep inside the cave) (Fig.-1a, b).

Figure- 1: Various zones of Kotumsar Cave from where the sediments were collected for isolation of *Deinococcus radiodurans*; a- entrance zone 'A' and transient zone 'B'; b- the deep inner zone 'C'

Screening of industrially important enzymes: as the first step, we prepared intracellular and extracellular crude enzyme extract from bacterial isolates followed by enzyme analyze process. All tested bacterial strains isolated from Kotumsar cave were screened for intra- and extra- cellular alkaline ptotease and glucose isomerase enzyme. Quantitative estimation were done by cystine/carbazole/sulphuric acid method (Dische & Borenfreund, 1951).

a) Alkaline protease: The quantitative estimation of alkaline protease was done by Nakagawa, (1970) method.

The alkaline protease activity was determined by the enzymatic hydrolysis of haemoglobin at alkaline pH. The liberated aromatic amino acid was measured in the trichloroacetic acid filtrate, which was allowed to react with Folin- Ciocalteu reagent, and the developed colour was measured at 750 nm.

The aliquot of 1ml of haemoglobin solution was equilibrated at 37°C for 5 min. The assay was initiated by adding 0.5 ml crude enzyme and mixture was incubated at 37°C for 10 min. At the end of incubation period the reaction was terminated by addition of 2 ml of 5% trichloroacetic acid (TCA). The suspension was allowed to stand for 5 min. and centrifuged at 5000 rpm for 20 min. In the control set, TCA was added before the enzyme addition, to stop the enzyme activity.

One ml supernatant was allowed to react with 5ml of alkaline solution for 10 min followed

by addition of 0.5 ml Folin Ciocalteu reagent and the colour developed after 15 min. was measured 750 nm in UV- visible spectrophotometer (Shimadzu). The specific activity was expressed as μ g tyrosine formed per mg protein per min.

b) Glucose isomerase: Quantitative estimation of xylose (glucose) isomerase was done by cysteine/carbazole/sulphuric acid method (Dische & Borenfreund, 1951)

The reaction mixture contained 1ml buffer solution, 0.5ml D-glucose and 0.5ml crude enzyme (extra- and intra- cellular). After incubation of the reaction mixture at 37°C for 15 min., 1 ml of 0.5 M perchloric acid was added to stop the enzyme reaction. The product was determined by the cysteine/ carbazole / sulphuric acid method. In this procedure, 1ml of supernatant (fructose formed from enzymatic action), 0.2 ml cysteine hydrochloride, 6ml H₂SO₄ and 0.2 ml carbazole solution were added sequentially, shaken and allowed to stand for 5 min. at room temp. Fructose gave purple colour and extinction was read against blank at 560 nm in UV- visible spectrophotometer (Shimadzu). The specific activity was expressed as μ g fructose formed per mg protein per min.

Statistical analysis: Two-way ANOVA was employed to verify the microhabitat effects on intra as well as extracellular enzyme; alkaline protease and glucose isomerase activities separately. Further the ANOVA results was followed by Duncan's Multiple Range Test (Duncan, 1955) to verify the statistically significance differences among every groups separately.

Results and Discussion:

(Results are summarized in Table 1a,b and Figure 2a,b)

Table-1a: ANOVA Summary for Alkaline Protease						Table-1b: ANOVA Summary for Glucose Isomerase					
Source	SS	df	MS	F	P	Source	SS	df	MS	F	P
Habitats	2157.46	2	1078.73	311.25	<.0001	Habitats	231.29	2	115.65	1186.1	<.0001
I Vs E*	181.9	1	181.9	52.48	<.0001	I Vs E*	278.76	1	278.76	2859.1	<.0001
Habitat X I Vs E*	1071.04	2	535.52	154.51	<.0001	Habitat X I Vs E*	237.38	2	118.69	1217.33	<.0001
Error	41.59	12	3.47			Error	1.17	12	0.1		
Total	3451.99	17				Total	748.6	17			

*Intra Vs Extracellular

(SS- sum of squares; df- degree of freedom, MS- mean square)

Inside the Kotumsar cave, the intra as well as extracellular enzyme activities of *Deinococcus radiodurans* for both alkaline protease and glucose isomerase were seen to be highly regulated with accordance to its ambient microhabitat conditions. Irrespective to both the studied enzymes, ANOVA results revealed very high statistically significant differences among all the employed parameters viz., habitats, Intra Vs Extracellular activities and also their

interactions. Further, the results from Duncan's multiple range test revealed high activity of Intracellular alkaline protease in strains; KCB21 and KCB93 i.e., the *D.radiodurans* strains isolated from the entrance 'A' and inner deep 'C' zones whereas the strain KCB50 isolated from the transient zone 'B' revealed very low activity of alkaline protease (Fig.-2a). Nevertheless, the extracellular alkaline protease production was seen to be increased with respect to the consistency of the cave environment i.e., the lowest alkaline protease activity was revealed from the strain isolated from the entrance zone 'A' than in the transient 'B' and highest was evidenced from the strain isolated from the inner deep zone 'C' (guano mix soil). Interestingly, extracellular alkaline protease activity of the isolated strain KCB93 isolated from the inner deep guano soil was also seen to be compatible to its intracellular counterpart (Fig.-2a).

Further, the intracellular glucose isomerase activity was found to be very low in the strain KCB50 isolated from the transient zone 'B', whereas its extracellular glucose isomerase activity was found to be compatible with the glucose isomerase activity revealed from strain KCB21, isolated from the entrance zone 'A'. However, both the extracellular and intracellular glucose isomerase activities revealed by the strain KCB93 isolated from the inner deep zone 'C' were found to be comparatively low (Fig.-2b).

Enzymes have been used since the beginning of the human civilization. Earlier it was restricted only to some domestic food processing phenomena, but later on i.e., from the twentieth century the concept of using enzyme as a detergent got momentum and enzyme production in bulk quantities started. When selecting a right strain to produce a desired enzyme, several aspects have to be considered which include high production in limited timeframe, required less purification process and optimum ambient conditions etc. The extracellular secretion of any enzyme is always preferable than its intracellular counterpart as, the recovery and purification process against extracellular enzyme productions is always much easier than its intracellular ones. It is mainly to avoid the various processes of purifications of the same from numerous types of cell proteins and other components associated with it (Leisola *et al.*, 2002). Hence, intensive screening programs are required to test a large number of strains for identifying the best producers that can yield high production along with novel properties.

Bacteria secrete proteases to digest (hydrolyse) the peptide bonds. Alkaline proteases are a very important group of enzymes due to its notable physiological and high commercial values. Primarily it is an essential requirement as detergent additives; in addition, it is also used in

Figure-2: Intra Vs extracellular enzyme activities of *Deinococcus radiodurans*. a- alkaline protease; b- glucose isomerase. Similar superscripts do not statistically differ from each other at 5% level.

leather processing, silver recovery, medical purposes, food processing, feeds, chemical industries and waste treatments as well. As it has already been discussed, being one of the toughest extremophilic bacteria, *D.radiodurans* is hard to isolate from common resources, thus the isolation of the same from various habitats of Kotumsar cave always enticed us to compare the enzyme production abilities with respect to their various habitats of occurrences. In the present study we found that the alkaline protease activity was high in the strain which was isolated from the guano mixed sediment of inner most zones “C” of the Kotumsar cave. Indeed, in extremophiles structural adaptations at the molecular level have already been proposed to cope up with the ambient exacting conditions. Earlier Rajput et al., (2012) stated that *D. radiodurans* strain KCB21, isolated from the sediment of entrance zone revealed limited protein bands, whereas the remaining strains of the same species isolated from deeper zones of the cave exhibited different specific protein bands of high molecular weights. In the present study, with regards to the apparent high potency of the strain KBC93 for extracellular alkaline protease activity, we can predict two possibilities for the same; a) the energy starved and almost constant ecological conditions of the inner deep zone of the cave increases the production of protease perhaps to hydrolyze more peptide bonds to survive b) various types of animal body parts remain as undigested materials in guano which leads to an increase in the protease activity in *D.radiodurans*. Due to energy starved conditions, various morphological, behavioural and physiological alterations in various bigger fauna abiding inside the Kotumsar cave have already been reported (Biswas, 1991; 2010; Biswas & Dey, 2014).

The most important role of Glucose isomerase is its interconversion of pentose and glucuronate as well as the metabolism of fructose and mannose. As an enzyme it has the largest market in the food industry because of its application in the production of high-fructose corn syrup (HFCS). In the present study, we found that the extracellular activity of glucose isomerase remains almost same in the strain isolated from the entrance “A” and the transient “B” zone (KCB21 & KCB50). Surprisingly the intracellular glucose isomerase activity of the strain KCB50 isolated from the transient “C” zone was revealed very low. Similarly, both the intra and extracellular glucose isomerase activity for the strain KBC93 isolated from the innermost “C” zone of the cave were also revealed statistically too low. This clearly shows that strains isolated from deeper habitats have a direct relation to decreasing levels of the glucose isomerase activity. However, when compared to the isolate of transient zone KCB50, less elevation in intracellular glucose isomerase activity was revealed in KBC93. This may be due to its growth in guano mixed soil that may contain undigested food materials of cave roosting bats.

Conclusion:

The *D.radiodurans* strain KBC93, isolated from the deep inner zone of the Kotumsar cave reveals high potency for extracellular alkaline protease which directly opens a window for searching of useful enzymes from more and more microbes existing in such extreme ecological niche.

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