Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 7 (2): 90-97, 2012
ISSN 1815-9923 / DOI: 10.3923/ajb.2012.90.97

Subterranean Depth Dependent Protein Constitutions of the *Micrococcus* sp. Isolated from the Kotumsar Cave, India

Yogita Rajput and Jayant Biswas
National Cave Research and Protection Organization, Central Zone, Raipur 492001, C.G., India

Corresponding Author: Jayant Biswas, National Cave Research and Protection Organization, Central Zone, Raipur 492001, C.G., India

ABSTRACT

The subterranean caves represent one of the most suitable examples of extremities in ecosystem and the microbes abiding in such environments represent truly extremophiles in nature. In the present study, *Micrococcus* bacterial strains were isolated from various depths of the Kotumsar cave (India) and further 12 strains of 3 specific species (*M. luteus*, *M. radiodurans* and *M. agilis*) have been selected for further studies. The protein profiles by SDS-PAGE technique were estimated for each strain and the effect of subterranean depth on the characterization of protein profiles have been tried to establish by the linear regression method. The strains isolated from the deeper zones of the cave exhibits more number of protein bands, carrying higher molecular weights. Statistical analysis also support the same i.e., the strains isolated from the deeper zones of the cave revealed much protein as compared to the strains isolated from the anterior zones. The chances of developing extremozymes (biocatalysts) in the strains, isolated from the deeper zones could not be denied. Further, the result obtained from this study also suggests redrawing the evolutionary tree of studied bacterial strains.

Key words: Extremophiles, SDS-PAGE, cave microbes, *Micrococcus* sp.

INTRODUCTION

The subterranean caves represent one of the most suitable examples of extremities in ecosystem for several particularities of biotic and abiotic factors. The high humidity and CO_2 concentration along with the constancy of other geophysical factors make the caves one of the most vulnerable environments in the Earth. Thus, the microbes living in such environments could be safely referred as extremophiles and the study of such microbes are always interesting for discovering potential microorganisms, important for several biotechnological tools.

Subterranean caves could be divided into various zones depending upon the characteristics of constancy of their immediate geophysical parameters: (1) The entrance zone i.e., the space around the cave entrance from where the inner zones of the cave remain in direct contact with the external environmental condition. (2) The twilight zone; nearer to the entrance, where light intensity, humidity and temperature varies according to its ambient environment. (3) The transition zone; almost complete dark with variable humidity and temperature. (4) The deep zone; complete dark with almost 100% humidity, constant temperature and almost complete detachment from the external environmental conditions (Aden, 2005; Biswas, 2009, 2010). Further, in addition to isolated environment, accumulation of various other internal factors viz., guano deposition, fungal colonies, biofilm/foval soil (Camassa, 1997) etc. have also been known to cause gradual alteration of cave habitat.

Kotumsar cave of India is the most biologically explored limestone cave of India (18°52'09" N; 81°56'05" E). Time to time several troglobiotic as well as troglophilic species have been reported from this cave (Biswas, 1992, 2010; Biswas *et al*., 2011). Till date the total passage mapped in the cave is nearly 660 m, out of which nearly 400 m is easily approachable. The roofs and walls of the different chambers are lined with colorful dripstone formations resulting from the precipitation of calcite-dissolved carbonate lime. The chambers of the cave are floored with either rocks or pebbles of various dimensions or by surface-derived soil/clay deposits. The cave is subject to frequent flooding during the monsoon season which usually begins in the middle of June and continues till the mid of October.

Screening of the bacterial populations inhabiting Kotumsar cave and updating the list is a continuous study being performed by us. Till date we have succeeded to isolate total 146 bacterial colonies from the soil samples collected from seven different depth and habitats dependent sites of Kotumsar cave. On the basis of PIB-win (probabilistic identification of bacteria) identification system, twenty one selected strains have been identified and confirmed with the help of ARDRA techniques (Thakur, 2009).

Microorganisms occupying heterogeneous habitats usually reveal apparent diversity in their biochemical characteristics. The characterizations of any available collection of microorganisms generally yield novel information regarding the nature of the existing gene pool and which could be varied in any parameter right from the morphological, biochemical, physiological to the molecular level during identification. Thus, the current conception suggests that the conventional tests based on the phenotypic characteristics may confuse the classification of some bacterial taxa. As per Murray *et al*. (1990), the electrophoresis technique, as a practical method, is necessary for integrated use of phenotypic characters in identification of bacterial genera at all level.

In the present study protein profiles of twelve selected strains belonging to three different species of *Micrococcus* were studied by using SDS-Polyacrylamide Gel Electrophoresis (PAGE) technique. Further, a linear correlation between the total number of protein bands exhibited by each strain and the average of molecular weight (kDa) revealed by each strain have been tried to establish separately with their respective subterranean depth of occurrences.

MATERIALS AND METHODS

Strains selection: Total 12 'PIB win' and 'ARDRA' based strains belonging to 3 different species of *Micrococcus* sp. (viz., *M. luteus*; *M. radiodurans* and *M. agilis*) were selected from isolated cultures for the study of protein profile. The strains of only those species were selected which were isolated at least from 4 different depth dependant habitats of the cave amounting to a total 12 strains (3 species X 4 habitats = 12) (Table 1).

Protein extraction: Bacterial cultures were grown overnight in LB broth (Luria-Bertani medium) pH 7, tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; distilled water, 1000 mL. 1.0 mL of bacterial broth was centrifuged at 5,000 rpm for 15 min in a microtube. The pellet was vertexed in sterilized normal saline and sonicated (Vibra Cell, USA) for 20 min at 50 kHz. Sonicated samples were again centrifuged at 15,000 rpm at 4°C for 30 min. The supernatant were passed through the syringe filter (Axiva, dia 2.5 cm, rating, 0.2 μm). Protein estimation was done by following Lowry *et al*. (1951) method. The purified protein solutions were mixed with sample buffer in the ratio of 1:2. The samples were taken in the Eppendorf tube and kept in boiling water bath for 5 min, centrifuged to eliminate the cell debris and used directly for electrophoresis.

Table 1: Isolated *Micrococcus* strains following PIB win scores and ARDRA support with respect to their habitat types, depth of occurrences

Code	Species isolated	Habitat type	Approximate depth of occurrences	Numerical code against depth	PIB WIN score	ARDRA support
1	*M. luteus* 4-KCB11	Entrance zone	2 m (from gate)	5	0.98	✓
2	*M. radiodurans* KCB21	Entrance zone	2 m (from gate)	5	0.92	✓
3	*M. luteus* 3-KCB38	Twilight zone	10 m (from entrance)	10	0.97	✓
4	*M. radiodurans* KCB39	Twilight zone	10 m (from entrance)	10	0.96	✓
5	*M. radiodurans* KCB82	Transient zone	45 m (from entrance)	15	0.92	✓
6	*M. agilis* KCB63	Transient zone	45 m (from entrance)	15	0.97	✓
7	*M. luteus* 1-KCB90	Deep zone (guano mixed soil)	80 m (from entrance)	20	1.00	✓
8	*M. radiodurans* KCB93	Deep zone (guano mixed soil)	80 m (from entrance)	20	0.96	✓
9	*M. agilis* KCB111	Deep zone (black soil)	105 m (from entrance)	25	0.99	✓
10	*M. luteus* 2-KCB113	Deep zone (black soil)	105 m (from entrance)	25	0.94	✓
11	*M. agilis* KCB140	Deep zone (laterite soil)	125 m (from entrance)	30	0.94	✓
12	*M. agilis* KCB125	Deep zone (foval soil)	140 m (from entrance)	35	0.99	✓

KCB: Kotumsar cave bacteria, 1: *Micrococcus luteus* 4-KCB11, 2: *Micrococcus radiodurans* KCB21, 3: *Micrococcus luteus* 3-KCB38, 4: *Micrococcus radiodurans* KCB39, 5: *Micrococcus radiodurans* KCB82, 6: *Micrococcus agilis* KCB63, 7: *Micrococcus luteus* 1-KCB90, 8: *Micrococcus radiodurans* KCB93, 9: *Micrococcus agilis* KCB111, 10: *Micrococcus luteus* 2-KCB113, 11: *Micrococcus agilis* KCB140, 12: *Micrococcus agilis* KCB125

Electrophoresis analysis of proteins: The protein profile of all the bacterial isolates were determined by SDS-polyacrylamide gel electrophoresis using 12.5% acrylamide gel and the marker following Laemmli's protocol (Laemmli, 1970). Ready-to-use protein molecular weight marker (Banglore Genei) was used for following the procedure and which was a mixture of purified protein as follows: myosin rabbit muscle, 205,00 Da; phosphorylase b, 97,400 Da, bovine serum albumin 66,000 Da; ovalbumin, 43,000 Da; carbonic anhydrase, 29,000 Da; soybean trypsin inhibitor, 20,100 Da; lysozyme, 14,300 Da; aprotinin, 6,500 Da; insulin, 3000 Da (2300-3400 Da).

Statistical analysis: To employ the statistical analysis first of all the observed results were quantified. The total number of protein bands (n) exhibited by each bacterial isolates taken as a parameter to verify the linear correlation between the obtained protein bands by each isolates and the subterranean depth (d) of its occurrences. The subterranean depth of each sample collection site were calculated (in meter) from the available map by graph measuring method. Though, the distances (in meter) of data collection sites were not equally distributed, to minimize the variances an equally distributed numerical code were assigned against each site i.e., 5, 10, 15, 20, 25, 30 and 35 (Table 1).

Further, linear correlation was also verified between the sum of total molecular weights (kDa) of the obtained proteins against each strain and their respective subterranean depth of collection site. To calculate it, the average molecular weights i.e., for each isolates the sum of molecular weight revealed by total number of exhibited protein bands were divided by number of total segaments of occurrence to quantify the average molecular weight of each strain revealed by SDS-PAGE analysis.

RESULTS

Cell filtrates protein profiles obtained by SDS-PAGE for 12 bacterial strains belongs to 3 specific species of Kotumsar cave are presented in Fig. 1 and Table 2.

Irrespective to the subterranean depth dependent sites of occurrences and the specific *Micrococcus* sp., the total numbers of clear protein bands were found to vary in between 8-21.

Table 2: Protein profiles of bacterial strains, isolated from different depth dependent habitats of Kotumsar cave, India

Segment	Entrance zone (2 m from gate)		Twilight zone (10 m vertical from entrance)		Transient zone (45 m)*		Deep zone (guano soil) (80 m)*		Inner zone (black soil) (105 m)*		Inner zone (laterite soil) (125 m)*	Inner zone (foval soil) (140 m)*
Code	1	2	3	4	5	6	7	8	9	10	11	12
1.	-	-	-	205	205	-	-	205	-	-	205	205
2.	-	-	-	-	-	-	-	-	-	-	-	115
3.	100	-	100	-	-	-	100	100	-	100	100	100
4.	-	99	99	-	-	-	-	99	99	-	99	99
5.	-	98	-	-	-	-	-	98	-	-	-	-
6.	-	97.4	97.4	-	-	-	-	97.4	97.4	-	97.4	97.4
7.	-	-	-	-	-	-	-	-	-	-	96	96
8.	-	-	-	90	90	-	-		-	-	-	-
9.	81.7	-	81.7	82	82	-	81.7	81.7	-	81.7	81.7	81.7
10.	80	-	80	80	80	-	80	80	-	80	-	-
11.	-	-	-	-	-	-	-	-	-	-	-	70
12.	66	-	66	-	-	-	66	-	-	66	66	66
13.	-	-	-	-	-	-	-	65	65	-	-	65
14.	-	-	-	-	-	-	-	-	-	-	-	64
15.	-	-	-	60	60	60	-	-	60	-	60	-
16.	-	56	-	-	-	-	-	-	-	-	-	-
17.	54.5	54.5	55	54.5	54.5	54.5	54.5	55	-	54.5	54	54.5
18.	-	-	-	-	-	-	-	-	-	-	-	50
19.	-	-	-	-	-	-	-	-	-	-	-	45
20.	-	43	43	-	-	43	-	-	43	-	43	43
21.	39	-	-	-	-	-	39	-	-	39	-	-
22.	-	-	36	36	36	-	-	-	-	-	36	36
23.	35	-	-	-	-	-	35	-	-	35	-	-
-24.	-	30	-	-	-	-	-	-	-	-	-	30
25.	29	-	29	-	-	29	29	29	29	29	29	-
26.	-	28	-	-	-	-	-	-	-	-	-	-
27.	26	-	-	-	-	-	26	-	-	26	-	-
28.	-	-	24.5	24.5	24.5	-	-	24.5	25	-	24.55	25
29.	20.1	20.1	20.1	-	-	20.1	20.1	-	20	20.1	20.1	20
30.	-	14.3	-	-	-	14.3	-	-	-	-	14.3	14
31.	6.5	-	6.5	-	-	6.5	6.5	-	-	6.5	6.5	6.5
32.	5	-	-	-	-	5	5	5	5	5	5	-
33.	4.7	-	-	-	-	4	4.7	-	-	4.7	-	-
34.	-	3	3	-	-	3	-	3	3	-	3	-
Total	547.5	543.3	741.25	632.05	632	239.4	547.5	939.6	446.4	547.5	1037.55	1383.1
Average#	16.1	15.98	21.8	18.59	18.59	7.04	16.1	27.63	13.13	16.1	30.52	40.68
No. of bands	13	11	14	8	8	10	13	13	10	13	18	21

Molecular weight (kDa) of bacterial protein *Distance from cave entrance. #Average n/∑z (total numbers of segments = 34) Bacterial isolates were as follows; 1: *Micrococcus luteus* 4-KCB11, 2: *Micrococcus radiodurans* KCB21, 3: *Micrococcus luteus* 3-KCB38, 4: *Micrococcus radiodurans* KCB39, 5: *Micrococcus radiodurans* KCB82, 6: *Micrococcus agilis* KCB63, 7: *Micrococcus luteus* 1-KCB90, 8: *Micrococcus radiodurans* KCB93, 9: *Micrococcus agilis* KCB111, 10: *Micrococcus luteus* 2-KCB113, 11: *Micrococcus agilis* KCB140, 12: *Micrococcus agilis* KCB125

Strains of *Micrococcus agilis*, isolated from the deepest zones of the cave exhibited highest number of bands, revealed high molecular weights; *M. agilis* KCB140 (bands-18, sum of mol. wts.-1037.55) *M. agilis* KCB125 (bands-21, sum of mol. wts.-1383.1).

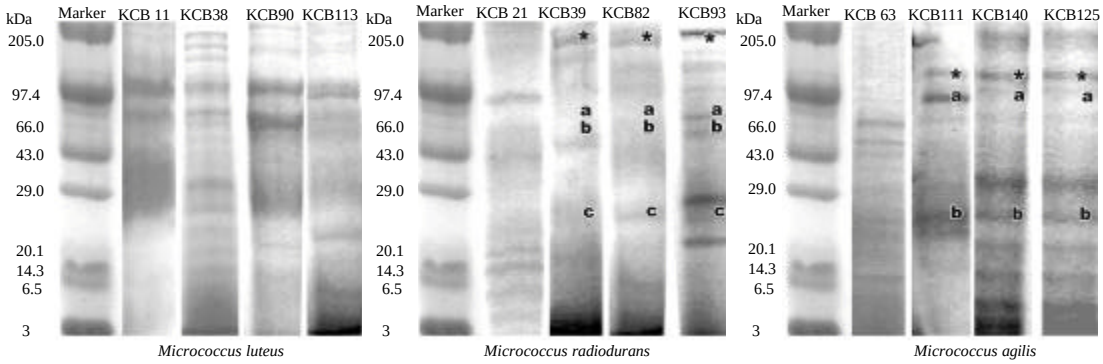

Fig. 1: A comparative perspective among marker and Protein bands exhibited by the isolated depth dependent strains of three *Micrococcus* sps. 'Script' on specific bands represents its respective molecular weight (kDa). (a) *Micrococcus radiodurans* (*-205 kDa; a-81.7-82 kDa; b-80 kDa; c-24.5 kDa) and (b) *Micrococcus agilis* (*-99 kDa; a-97.4 kDa; b-24.5-25 kDa)

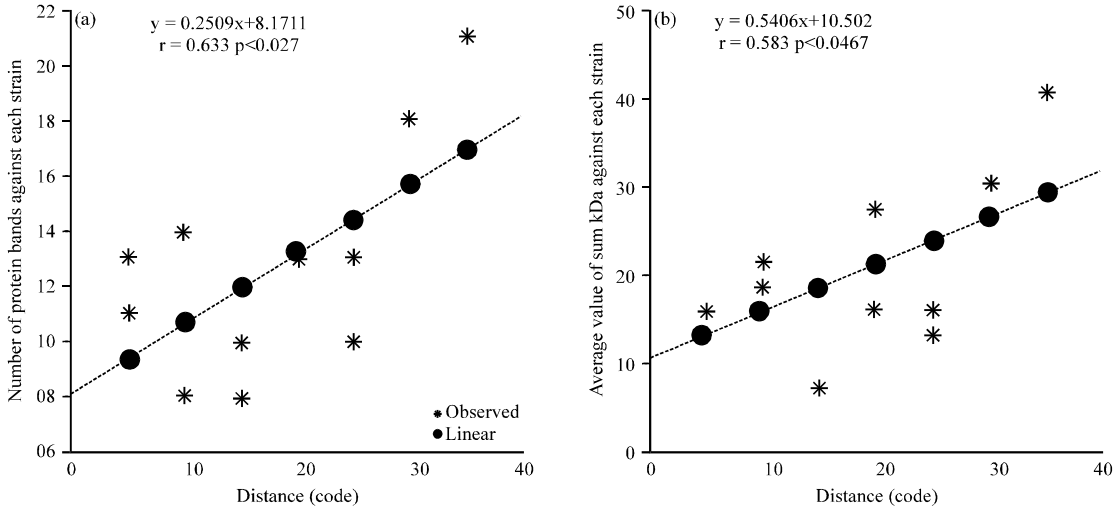

Fig. 2(a-b): (a) A positive linear correlation between the number of protein bands exhibited by each strain of *Micrococcus* sp. during SDS-PAGE electrophoresis and their respective subterranean depth of occurrences and (b) A positive linear correlation between the average of total molecular weight (kDa) of the protein bands exhibited by each strain of *Micrococcus* sp. during SDS-PAGE electrophoresis and their respective subterranean depth of occurrences

Comparative chart clearly indicate that protein band variations were mostly restricted to the molecular proteins having lower molecular weights. However, excluding the strain 3-KCB38 of *Micrococcus luteus,* isolated from the twilight zone, rest 3 other strains of the same species isolated from three different subterranean depth dependent habitats neither exhibited any difference in their total sum of obtained molecular weight of the protein bands nor in the total numbers of protein bands revealed from SDS-PAGE electrophoresis. Nevertheless, the remaining 8 strains belonging to 2 different species (viz., *Micrococcus radiodurans; Micrococcus agilis*) revealed apparent depth dependant variations (Table 2; Fig. 1, 2).

Further, the total number of protein bands obtained against each strain evidenced positive correlation with their respective subterranean depth of occurrences ($r = 0.633$; $p<0.027$). More or less the same result was seen when the linear correlation was employed between the average value of total sum of molecular weight and the subterranean depth of their respective occurrences ($r = 0.582$; $p<0.046$). The result simply revealed that the strains which were isolated from the deeper zones i.e., more extreme conditions were having more protein and with higher molecular weights whereas the strains isolated from such zones which more or less remain under the influence of external environmental conditions were with low protein profiles.

DISCUSSION

In extremophiles a structural adaptations at the molecular level have been proposed to cope up with the ambient exacting conditions. Further, some extremozymes (biocatalysts) which are produced by these microorganisms have been shown to play the key role to withstand such harsh conditions (Kuddus *et al.*, 2011). In our study, the strains which were isolated from the deeper zones revealed few additional specific protein bands which were altogether absent in their remaining counterparts; isolated from the anterior (nearer to the entrance) zones of cave. The strain; KCB21 of *Micrococcus radiodurans*, isolated from the soil sample of entrance zone did not show any specific band in its 1st segment of protein profiles, whereas the remaining 3 strains of the same species isolated from the deeper zones of the cave exhibited specific protein bands (mol. wts. 205 kDa). The same patterns were repeated in further 9th, 10th as well as in 28th segments. Not a single band was revealed in those strains of *M. radiodurans* which were isolated from the entrance zones whereas the other remaining strains, isolated from the deeper zones revealed specific protein bands (segment 9:-81.7-82 kDa; segment 10:-82 kDa and segment 28:-24.5 kDa).

While analyzing the protein profiles of various strains of *Micrococcus agilis*, isolated from different depth dependent habitats, we found some interesting results, revealing almost similar prototype. During our study we failed to isolate any strain of *M. agilis* from the entrance and/or other anterior zones of the cave. The strain; KCB63 of *M. agilis* which was isolated from the anterior most (transient) zone of the cave was also found to deviate enough in the prototype of protein profiles from its counterparts, isolated from the deeper zones of the cave. The strain KCB 111, KCB140 and KCB125 of *M. agilis*, isolated from the innermost zones of the cave revealed some extra bands at the protein profile segments-4 (kDa-99), 6 (kDa-97.4) and 28 (kDa-24.5 to 25) which were altogether lacking in the strain KCB63 of *Micrococcus agilis*. Absence of this protein bands in organisms living in transient zone possibly attributable to the external environmental conditions of the cave as the transient zone of the cave is under the influence of the external environmental conditions to a limited extent.

Total 4 *Micrococcus luteus* strains named; *Micrococcus luteus* 4, from external zone, *Micrococcus luteus* 3, from twilight zone and *Micrococcus luteus* 1 and 2 from two different habitats of the deeper zone were isolated, identified by PIB win and ARDRA methods. Surprisingly, while analyzing the protein profiles of all these strain we found the strains isolated from the entrance and the deeper zones are almost alike, where as the strain isolated from the twilight zone revealed dissimilarities in positions and numbers in the occurrences of protein bands. Although, the specific reason for such differences among all the strains of this particular species revealed by PIB Win identification method is yet to be elucidated, the subterranean depth was found to exert almost nil effect in the spectrum of protein profiles of the same.

Bacterial community which was discovered from the depths of more than a kilometer was found to play a key role in geochemical processes and thus provoked the cave-microbiologists to search for those microbes which might play a major geochemical role as well (Culver, 2005). In the present study, recurrence of some additional protein bands in the strains isolated from the deeper zones of the cave possibly offering paramount importance for understanding the effects of extreme environment on living organism as this part of the cave always represent an extreme condition. Characterization of these extreme conditions' proteins produced by the respective resident microbes for their survivability can be exploited for several biotechnological means. Nevertheless, studies of extremophiles have also helped to redraw the evolutionary tree of life (Madigan and Marrs, 1997) Conclusively, a further study of these extremophile proteins produced by microbes of the deeper zones of Kotumsar cave will certainly not be restricted to the beneficial of its utilities in several biotechnological tools but also throw sufficient light for reconstruction of evolutionary tree of life.

ACKNOWLEDGMENT

We are highly grateful to the Director, Kanger Valley National Park for allowing us to collect the samples. 1st author (YR) is highly grateful to the Head, School of Life Sciences, Pt. Ravishankar shukla University, Raipur for allowing her to conduct the laboratory work. Thanks are also due to Prof. R.K. Pradhan, School of Life Sciences, Pt. Ravishankar Shukla University, Raipur, India for his valuable suggestions and critics for the earlier version of the current manuscript.

REFERENCES

Aden, E., 2005. Adaptation to Darkness. In: Encyclopedia of Caves, Culver, D.C. and W.B. White (Eds.). 1st Edn. Elsevier Academic Press, Burlington, CA., USA., ISBN-13: 978-0121986513, pp: 1-3.

Biswas, J., 1992. Kotumsar cave ecosystem: An interaction between geophysical, chemical and biological characteristics. NSS Bull., 54: 7-10.

Biswas, J., 2009. The biodiversity of Krem Mawkhyrdop of Meghalaya, India, on the verge of extinction. Curr. Sci., 96: 904-910.

Biswas, J., 2010. Kotumsar cave biodiversity: A review of cavernicoles and their troglobiotic traits. Biodiversity Conservation, 19: 275-289.

Biswas, J., S. Shrotriya, Y. Rajput and S. Sasmal, 2011. Impacts of ecotourism on bat habitats in caves of Kanger Valley National Park, India. Res. J. Environ. Sci., 5: 752-762.

Camassa, M., 1997. Biological observations in an artificial cave of Ginosa (Southern Italy): Role of the foval in cave ecosystems. Thalassia Salentina, 23: 189-191.

Culver, D.C., 2005. Microbes. In: Encyclopedia of Caves, Culver, D.C. and W.B. White (Eds.). 1st Edn. Elsevier Academic Press, Burlington, CA., USA., ISBN-13: 978-0121986513, pp: 369-371.

Kuddus, M., Roohi, J.M. Arif and P.W. Ramteke, 2011. An overview of cold-active microbial α-amylase: Adaptation strategies and biotechnological potentials. Biotechnology, 10: 246-258.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Madigan, M.T. and B.L. Marrs, 1997. Extremophiles. Sci. Am., 276: 82-87.

Murray, R.G.E., D.J. Brenner, R.R. Colwell, P. Devos and M. Goodfellow *et al.*, 1990. Report of the ad hoc committee on approaches to taxonomy within the proteobacteria. Int. J. Syst. Bacteriol., 40: 213-215.

Thakur, Y., 2009. Isolation and characterization of bacteria from Kotumsar cave of Chhattisgarh. Ph.D. Thesis, Pandit Ravishankar Shukla University, Raipur, India.