DIVERSITY OF EXTREMOPHILIC MAGENSITE BACTERIA AND IT'S ENZYMATIC POTENTIAL

Ramya Suseenthar[1], Shanthi John[2] and Balagurunathan Ramasamy[3]

Periyar University, Department of Microbiology, Periyar University, Salem-636011
rbalaguru@yahoo.com

ABSTRACT

The present study focuses on the diversity of extremophilic bacteria from magnesite soil which are less explored and screened for the multitude of commercially important enzymes produced. Five different soil samples varying in color were collected from magnesite deposit Salem, Tamil Nadu. The soil samples had pH of 8-9, high nitrogen content, low phosphorous, potassium and trace elements. A remarkable reduction in total heterotrophic bacterial diversity of the ranging between 2.0x 10^7 CFU/g and 5.2x10^5 CFU/g. Generic composition of 25 isolates comprised of 28% *Bacillus* spp., followed by 20% *Staphylococcus* spp., 16% *Enterobacter* spp., 12% *Pseudomonas* spp., 12% *Escherichia coli*, 8% *Micrococcus* spp. and 4% *Serratia* spp. Maximum of 72% of isolates showed lipase activity followed by 68% L-asparaginase, 60% protease, 52% cellulase, 44% L-glutaminase, 20% amylase and 4% tyrosinase activity were observed. The dialysate of culture filtrate exhibited 27000 U/ml of lipase, 5393 U/ml and 5923 U/ml of L-asparaginase and 4074 U/ml of α-amylase activity. The 16s rDNA sequence analysis of S5B2 showed 100% similarity with *Bacillus pumilus* (X8), S1B4 with 99% of *Bacillus pumilus* (X22) and 99% of S2B2 with *Bacillus licheniformis* (2J-1). This is the first report on the bacterial diversity from Magnesite soil with the potential to produce industrially robust enzymes for various biotechnological applications.

Keywords: Magnesite soil, bacterial genera, heterotrophic population, enzymes.

INTRODUCTION

Diversity is composed of richness and evenness and it occur in nature across all forms of life at global, regional and local scale. Researchers concentrate mainly on genetic diversity in natural population of microorganisms, plants, animals and humans (Nevo, 2001). Microorganisms represent the richest repertoire of molecular and chemical diversity in nature, as they comprise the most diverse form of life and also they are extra ordinary reservoir of life in the biosphere that we have to explore and understand (Bhavdish *et al.,* 2005). The diversity of microbial populations in extreme habitat is greatly influenced by various physico-chemical characteristics of that particular ecosystem. During the past decade there has been a large increase in search of organisms existing at extreme conditions, driven by the realization that they have a great potential for remarkable application in agriculture, industry, medicine and environment (Bhavdish *et al.,* 2005; Nicholas Russell, 2006). Among the extreme microorganisms, bacteria are the most dominant group involved in the biogeochemical cycling mainly mediated by their enzymatic reactions.

The core of traditional biotechnology is the usage of microbial enzymes and three thousand enzymes which catalyze a wide variety of chemical reactions are known, but only few enzymes from the extreme environmental organism were used in industrial scale (Andreea Oarga, 2009).The potential of microorganisms as source of industrially relevant enzymes has stimulated a renewed interest in the exploration of microorganisms for extra-cellular enzyme activity from rare extreme environment. Enzymes from rare environment may exhibits more stability and also economically very important. The chemical industries are in need of different enzymes stable to high temperature, pH, ion strength, etc (David *et al.,* 1999). This has started a search for naturally occurring enzymes that is intrinsically stable to a broad range of parameters from organisms surviving in harsh condition by producing novel enzymes called extremozymes. Magnesite mine in Salem is one of such harsh condition have not yet been explored in terms of microbial diversity and prospecting. Magnesite mines are widely distributed throughout the world but have not been explored for the microbiological point of view. In this study we screened the bacterial diversity, characterized the enzymatic potential of isolates and identified the potential bacteria species.

MATERIALS AND METHODS

Study Area and Sampling Procedure

The study area Magnesite deposit, Salem, (Latitude 11° 40' - 11°46' N; Longitude 78º5' - 78º11' E) Tamil Nadu .Soil samples were collected from Salem magnesite deposit areas in five different sites, these five sites were chosen as a representative of five different type of soil based on their colour, texture, at the depth of 1cm. All the samples are named as M1, M2, M3, M4, and M5 collected in sterile polythene bags and transported to the laboratory for analysis.

Soil samples were analyzed for the physico-chemical characters pH, lime status, texture, nitrogen, phosphorous, potassium, copper, magnesium, ferrous and zinc by adapting standard methodology.

Enumeration and Identification of Bacteria

Enumeration was carried out by standard serial dilution technique using Nutrient agar for bacteria and incubated at 28°C for ten days. The plates containing 30 -300 bacterial colonies were selected for enumeration and the population were expressed as number of colony forming units per gram (Cfu/gm). Well isolated bacterial colonies with varying morphological characteristics were streaked in nutrient agar plates and the selected strains genus identified using Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

Screening of Bacteria for Enzymatic Activity

All the twenty five strains were subjected to primary screening of the enzymes α-amylase, protease, lipase, cellulase, L-asparaginase, L-glutaminase and tyrosinase activity by spot inoculation method. Screening of isolates for amylolytic activity was carried out by growing the organisms on a nutrient agar medium plate containing 1% starch and subsequently staining it with iodine vapor. All the bacterial strains were screened for proteolytic enzyme production on skim milk agar medium. The inoculated plates were then incubated at 37°C for 48 hrs. Selection of lipolytic producing organisms was made after streaking 0.1 ml bacterial cultures on nutrient agar plates containing 1.0% (v/v) tween 80 and supplemented with 0.1% yeast extract, 0.01% $CaCl_2$ and 1.5% Na_2CO_3. Cellulase activity of the isolates was identified by cellulose agar plate (Teather and Wood, 1982). All the twenty five isolates were stabbed on the solidified agar and allowed to incubation for 2 days to express cellulose depolymerization through cellulase production in the surrounding medium. All the cultures were qualitatively examined for the production of enzymes L- asparaginase, glutaminase and tyrosinase using mineral salt agar plate supplemented with 1% L-asparagine, glutamine and tyrosine respectively.

Enzyme Production in Liquid Media

Bacterial isolates with the highest enzymatic activity in the primary screening was selected for further production. The enzyme production was carried out by adapting shake flask fermentation method. The medium used for production was tween 80 broth with 1% tween 80 (Anjana Sharma *et al.,* 2009) for lipase, mineral salt broth with 1% L-asparagines for L- asparaginase and nutrient broth with 1% starch (Manoj Agrawal *et al.,* 2005) for amylase. The media were inoculated with 1 ml of an overnight culture and incubated in a rotary shaker at 120 rpm for 48 hours at 28°C. At regular intervals, the triplicate samples were harvested and the cells were separated by centrifugation at 10,000 rpm for 20 min. The cell-free culture supernatant collected from each of the medium was used to determine the amount of enzyme liberated by the organisms.

Ammonium Sulphate Precipitation and Dialysis

Enzymes from the fermented broth for lipase, L-asparaginase and amylase were precipitated using ammonium sulphate (Sabu *et al.,* 2005). Finely powdered ammonium sulphate was added at 20% saturation and allowed to stand for 30 min at 4ºC. The precipitate obtained was separated by centrifugation and the resulting supernatant was further treated with ammonium sulphate at 40% saturation. The precipitate obtained was collected by centrifugation. The supernatant was similarly treated with ammonium sulphate at 60 and 80 % saturation and the precipitates were obtained. All the precipitates were resuspended in a minimal amount of buffer (0.01M Phosphate buffer, pH 8.0). The precipitated protein obtained from the ammonium sulphate precipitation in the form of pellet was dissolved in 0.01M buffer and dialyzed using dialysis membrane overnight against phosphate saline buffer at 4°C and the partially purified enzymes were quantified using enzyme assay (Distasio *et al.,* 1976).

Enzyme Assay

Activities of lipase, L-asparaginase and amylase were assayed using culture supernatants. Lipase activity was determined in a reaction mixture containing 5 ml of olive oil emulsion, 2 ml of 0.05 M phosphate buffer (pH 8.0) at 37^{o}C for 30 minutes in a water bath. The liberated fatty acid was titrated with 0.05N sodium hydroxide using phenolphthalein as indicator (Abramic *et al.,* 1999). L-asparaginase activity was determined in 1.5 ml reaction mixture containing 0.5 ml of asparagine, 50M phosphate buffer (pH8.0) incubated at 37^{o}C for 30 minutes. The reaction was stopped by adding 0.1 ml of 15% trichloroacetic acid. The supernatant obtained after centrifugation at 6000 rpm for 5 minutes was added to 6 ml of Nessler's reagent and optical density was measured at 480 nm (Wriston and Yellin, 1973). Amylase activity was determined in the reaction mixture containing 1ml of soluble starch, 1 ml of enzyme and incubated for 15 minutes. The reaction was stopped by adding 2 ml of dinitrosalicyclic acid (DNS reagent) and the solution was boiled for 5 minutes. About 1 ml of potassium tartarate was added and the reaction mixture was made up to 10 ml with distilled water and absorbance was measured at 560 nm (Bernfield *et al.,* 1958).

16s rDNA Sequencing

Genomic DNA of the bacteria was extracted using phenol-chloroform and agarose gel electrophored, for verifying DNA quality. The gene that encodes the 16s rRNA was subjected to PCR (Polymerase Chain Reaction) amplification using the kit big dye terminator version 3.1 cycle sequencing kit and 2 games of universal primers; 16s for 27A 5′AGAGTRTAGATCMTYGCTWAC 3′and 16r1488 5′CGYTAMCTTWTTACGRCT 3′. The PCR amplified DNA fragments were purified and sequenced by both strands in an automatic sequence, AB 13130 genetic analyzer (Chromus Biotech, Bangalore, (Chun Bae *et al.,* 2000).

Phylogenetic Analysis

The sequence was assembled using the "sequencer" program, edited with the bioedit program and then compared with the gene bank database (http://www.ncbi.nlm.nih.gov / gene bank / index.html). BLASTN program was used to search sequences deposited in gene bank. Subsequently, the program CLUSTALW was used to align the sequence from the database and with the help of mega software program the phylogenetic tree was visualized (Thompson *et al.,* 1994).

RESULTS AND DISCUSSION

Physico chemical analyses of five soil sample are illustrated in Table 1. In all the five samples analysed, nitrogen was high and potassium and phosphorous was less, trace elements like copper, magnesium, ferrous and zinc level in the soil was even lesser. The analysis of five soil sample from Salem Magnesite mine revealed that the carbon was available as bicarbonate; nitrogen content was high where as potassium and phosphorus was less. However the elements like copper, magnesium and silica was only present trace inquantity. In addition magnesite soil also contained low concentration of silicon oxide (Sio_2) – 2.38%, aluminum oxide ($Al_2O_{3)}$ – 0.10%, ferric oxide (Fe_2O_3) and ferrous oxide (Feo) -0.06%, calcium oxide (Cao) -0.42%, magnesium oxide (MgO) -46.35% (Andreea Oarga, 2009). Soil analysis clearly indicates that the nutrients, pH, temperature and humidity were different from the normal ecosystem as a potential rich source for isolation of rare extermophilic micro organism that is less explored. The main factor that implies the bacterial population depends upon the type of nutrients available, moisture, temperature (Prabakaran, 2004).

The total heterotrophic bacterial populations in soil samples are represented in Table 2. The total viable count of bacteria ranged only from $5.2x10^4$ Cfu/g and $2.0x10^5$ Cfu/g. Well isolated bacterial colonies with varying morphological growth characterization were selected for identification (Fig 1) and about twenty five such colonies were selected for the study. About 7 bacterial isolates belongs to the genus Bacillus spp., 5 to *Staphylococcus* spp. , 4 to *Enterobacter* spp. *Pseudomonas* spp. and *Escherichia coli* each comprised of 3 isolates, *Micrococcus* spp. of and *Serratia* spp. 1 isolate.

Out of twenty five bacterial isolates subjected to primary screening (Table 3), a single *Bacillus* spp., (S1B2) strain harbored six enzymes production capability (amylase, protease, lipase, cellulase, asparaginase and Glutaminase). Generic compositions of the 25 microbial strains imply their key role in soil process that determines plants productivity.

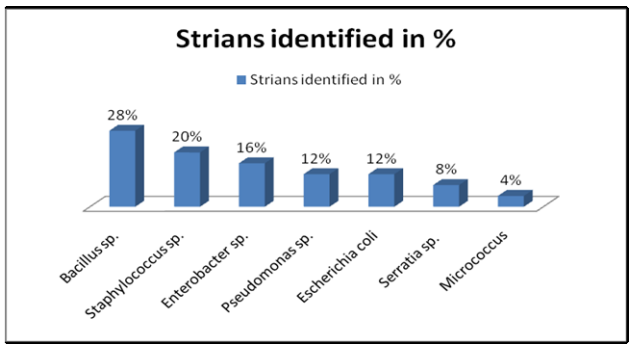
For successful functioning of microbes and their influence on mine processing, exhaustive efforts have been made to explore soil microbial diversity of indigenous community (Hill, 2000). Five isolate, belonging to genus *Bacillus* spp. (S1B4), *Escherichia coli* (S1B3) and *Staphylococcus* spp. (S1B1, S4B4 and S5B1) produced five enzymes. Four strains, of *E.coli* (S2B1, S4B3) and *Enterobacter* spp. (S2B3, S4B5) had four enzymes.

Table 1: Physico-chemical analysis of soil samples collected from magnesite soil

Parameters	Samples				
	M1	M2	M3	M4	M5
pH	8.4	8.1	8.6	8.6	8.6
Lime status	Medium	Medium	Medium	Medium	Medium
Texture	Loam	loam	Loam	Loam	Loam
Nitrogen (%)	71	74	32	45	76
Phosphorus (%)	11	11	11	4	9
K (%)	7	7	7	7	21
Cu (%)	3.2	3.6	-	-	1.6
Mg(%)	2.2	2.4	0.2	0.18	1.24
Fe (%)	0.12	0.14	0.14	0.14	0.16
Zn (%)	0.36	0.08	0.12	0.16	0.16

Three strains *Bacillus* spp. (S3B3) *Staphylococcus* spp. (S5B3) and *Enterobacters* spp. (S3B4) does not have capability to exhibit activity against all the seven enzymes.The potential strains enzymes productions are listed Table 4. The microorganisms survive the harsh condition by producing novel stable industrially important enzymes. Few *Bacillus* spp. from magnesite soil had ability to produce amylase (Suman and Ramesh, 2010) where as others and *Pseudomonas* spp.*, Staphylococcus* spp. had protease production capability at pH 8 (Mussarat Shaheen *et al*., 2008).

Figure 1: Generic composition of the bacterial isolates



There are various reports on the production of enzymes lipases, L-asparaginase, L-glutaminase from *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *E.coli, Erwinia cartovora, Enterobacter aerogenes, Corynebacterium* sp., *and Staphylococcus aureus* (Ahmed *et al.,* 2003; Madan and Mishra, 2010; Prakasham *et al.,* 2010). Cellulase activity was exhibited by 52% of the total bacterial strains and was active at high pH (Ito, 1997). Lipase enzyme production by S5B2 was maximum at 48 hours. The production decreased after 48 hours due to the depletion of carbon and lipase enzyme activity of 27000 U/ml was measured. The partially purified dialysate gave lipase activity was 31500 U/ml. L- Asparaginase activities of two bacterial strains S1B4 and S2B2 showed maximum activity at 48 hours of incubation. The enzyme activity produced by dialysate was 5393 U/ml (S1B4) and 5923 U/ml (S2B2) observed higher than the crude–1331 and 1997U/ml enzyme. Maximum enzyme activity of 1110 U/ml for amylase was observed at 48 hours and the activity was increased to 3074 U/ml after precipitation by ammonium sulphate further the dialysate culture filtrate contained amylase activity as 4140 U/ml. Enzyme produced by submerged fermentation increased continuously and its maximum production was obtained at 48 hours (Vidhyalakshmi *et al.,* 2009). It decreased after 48 hours due to the depletion of nitrogen and other nutrients being utilized or by inactivation of enzyme by acidification of the medium (Dercova *et al.,* 1992).

Table 2: Total Bacterial count in soil samples

Sample	Bacterial count Cfu/gm
M 1	2.0×10^5
M 2	4.3×10^4
M 3	5.2×10^4
M 4	4.3×10^5
M 5	3.2×10^5

Table -3: Enzymatic potential of twenty five strains

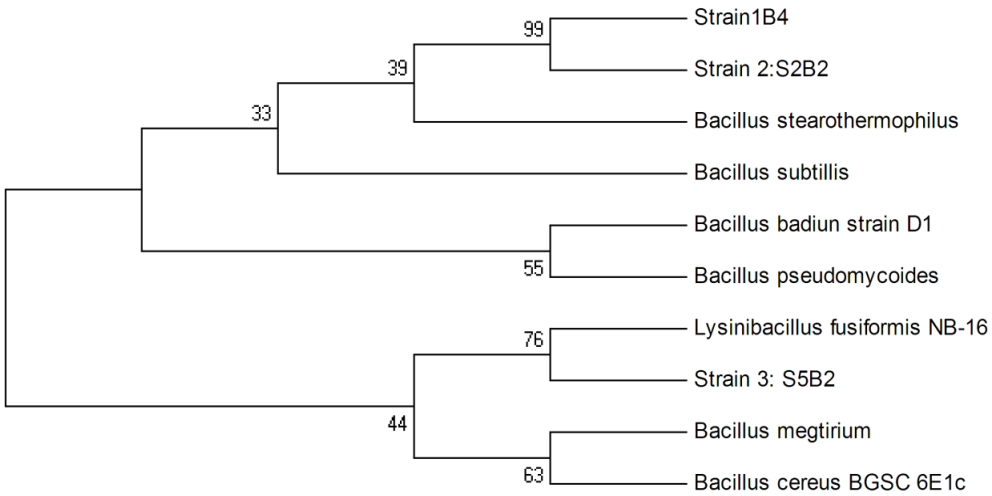
Strain number	Amylase	Protease	Lipase	Cellulase	Asparaginase	Glutaminase	Tryosinase
S_1B_1	-	+	+	+	+	+	-
S_1B_2	+	+	+	+	+	+	-
S_1B_3	+	+	-	+	+	+	-
S_1B_4	-	+	+	+	+	+	-
S_2B_1	-	+	+	+	+	-	-
S_2B_2	-	+	+	-	+	-	-
S_2B_3	-	+	+	-	+	+	-
S_2B_4	+	+	+	-	-	-	-
S_3B_1	-	+		+	-	-	-
S_3B_2	-	+	+	+	-	-	-
S_3B_3	-	-	-	-	-	-	-
S_3B_4	-	-	-	-	-	-	-
S_3B_5	-	-	+	-	+	-	-
S_4B_1	-	-	+	+	+	-	-
S_4B_2	-	-	+	-	+	-	-
S_4B_3	-	+	+	+	-	+	-
S_4B_4	+	-	+	+	+	-	+
S_4B_5	+	-	+	+	-	+	-
S_5B_1	+	+	+	-	+	+	-
S_5B_2	-	+	-	-	+	+	-
S_5B_3	-	-	-	-	-	-	-
S_5B_4	-	+	-	+	-	-	-
S_5B_5	-	+	+	-	+	-	-
S_5B_6	-	-	+	-	-	-	-
S_5B_7	-	-	-	+	-	-	-

+ - positive, - - Negative

Table 4: Assay of Lipase, L-Asparaginase and Amylase

Culture filtrate purification steps	Enzymes			
	Lipase	L- Asparaginase		Amylase
	S5B2	S1B4	S2B2	S4B4
24 hours	750	865	332	46
48 hours	2250	1,997	1,331	1,110
Protein precipitant	27,000	2,663	5,327	3,074
After dialysis	31,500	5,393	5,923	4,140

Figure 2: Phylogenetic analysis of bacterial isolates



Lipase enzyme produced by S5B2 was precipitated using ammonium sulphate precipitation and the yield was increased (Gupta *et al.,* 2004). In the present study lipase production by *Bacillus pumilus* S5B2 was 31500 U/ml it is comparatively higher than the lipases of *Bacillus pumilus F*3 of 483 U/ml as reported (Kambiz *et al.,* 2008). L-asparaginase production and its partially purified dialysate culture filtrate of *Bacillus licheniformis* S2B2 was 5923 U/ml was also higher than the previous reports in the production of extracellular L-asparaginase by *Bacillus* sp*.,* (Kei Arima *et al.,* 1972). Maximum amylase activity of 1110 U/ml after 48 hours was recorded in the present study similar to amylase production by *Bacillus flavothermus* (Manoj Agrawal *et al.,* 2005).

The phylogenetic tree of 16 s rRNA sequence obtained from PCR amplification is represented (Fig 2). Isolates S1B4, S2B2, and S5B2 was associated with the members of the diverse *Bacillus* spectrum showing sequence similarities of 99%. Strain S1B4 had similarity with *Bacillus pumilus* (X22*),* S2B2 with 99% of *Bacillus licheniformis* (2J-1) and 100% similarity of S5B2 with *Bacillus pumilus* (X8). The sequence was submitted in the database with accession number JN166722, JN166721 and JQ843918.

The 16s rRNA gene has been usually used as a trustworthy molecular marker for phylogenetic analysis of organism, it contains conserved region a unique array of sequence that are relative among species or different species (Moyer *et al.,* 1994). It could be seen from the topology of the built in phylogenetic tree that there was a considerable phylogenetic distances exist between *Bacillus pumilus* (S1B4) *, Bacillus licheniformis* (S2B2) and *Bacillus pumilus* (S5B2) in nucleic acid levels with other *Bacillus* sp. The tree shows that strain S1B4, S2B2 and S5B2 was separated from high homology standard sequence of different *Bacillus* genus and species of *Bacillus stearothermophilus, Bacillus subtilis, Bacillus badium strain D1, Bacillus Pseudomycoides. Lysinibacillus fusiformis NB-16, Bacillus megtirium and Bacillus ccereus* BGSC 6EK. Based on the similarities of the gene sequence, the strains S1B4, S2B2 and S5B2 were identified as *Bacillus pumilus* (S1B4)*, Bacillus licheiformis* (S2B2) and *Bacillus pumilus* (S5B2).

CONCLUSION

Screening of microbial strains from the magnesite mines deserves special attention to explore the vast potentialities of endemic and diverse microflora for their commercial usage. On the basis of the present finding it is emphasized that biodiversity of organisms depend upon habitat with the inherited physiological, biochemical and generic composition of the micro organism. Screening and production of enzymes from magnesite soil suggests that it contains considerable number of extra cellular enzymes of industrial importance. In fact production and purification by dialysis proved that the isolates produced considerable high amount of the enzyme. Hence this study strongly recommends the importance of the magnesite soil as a potential source of valuable enzymes for industries.

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