



Original Research Article

Extra Cellular Enzyme Production by *Actinobacillus*: A thermophile present in cattle compost

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A B S T R A C T

Keywords

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High temperature exerts a selection pressure which turns a microorganism to a thermophiles and thermo stability is a characteristic of most of the enzymes available from these microorganisms. Thermophilic microorganisms are of special interest as a source of novel thermostable enzymes. Enzymes stable at higher temperature has an immense significance in various industries. A total of 20 bacterial strains, isolated from cattle compost and believed to be thermophiles were screened for the extracellular enzyme production including amylase, lipase, cellulase and protease. The study was designed for characterization of the properties which are responsible for their adequate nature to sustain high temperature. Research finding revealed the production of extracellular enzymes by these isolates. Microorganisms were isolated from cattle compost and initially screened by biochemical tests to characterize till genus level. Confirmation of *Actinobacillus spp.* was done by fermentation reactions of nine carbohydrates and amplification of 16S rRNA region. Assays were designed to confirm the production of extracellular enzymes. Heat shock protein profiling was also done for the selected isolates to find out their important role in high temperature conditions. This study initially designed to study the diversity of thermophiles present in compost which turned specifically to *Actinobacillus*. *Actinobacillus* isolates was shown to be positive for various extracellular enzyme production. Study of heat shock protein and protein profiling suggested that these protein can show a high temperature dependent variation.

Introduction

Environment conditions are important factor which impact a lot on the survival of any organism and out of this temperature is the most vital. Moderate environments is near neutral pH, temperature between 20 to 40°C, air pressure 1 atm and adequate levels of

available water, nutrients and salts. Any environmental condition that can be perceived as beyond the normal acceptable range is an extreme condition. However, a variety of microorganisms survive in those conditions also. These organisms not only

tolerate but require such conditions for their growth and survival. Such organisms are called extremophiles (Satyanarayana, 2005). Of these extremophiles, organisms which survive in high temperature areas are called thermophiles. Some species of thermophiles can even survive at the elevated temperature of hot spring. Thermophiles can be further classified as Obligate, which require high temperature for their survival, and moderate, which can thrive at high temperatures but also at lower temperature. For an organism to grow at high temperatures, especially as high as those of the hyperthermophiles discussed here, all cellular components, including proteins, nucleic acids, and lipids, must be heat stable (Brock, 1967; Brock et al., 1970). The thermal stabilities of enzymes from various hyperthermophiles are referred as extremozymes (Gomes et al., 2004) and some of such enzymes have been found to remain active up to 140°C (Ladenstein et al., 1998).

Besides temperature, other environmental parameters such as pH, available energy sources, ionic strength and nutrients also influence the population of thermophiles. The structural features that dictate thermal stability in proteins are not well understood but a small number of noncovalent features seem characteristic of thermostable proteins. These include a highly apolar core, which undoubtedly makes the inside of the protein "sticky" and thus more resistant to unfolding, a small surface-to-volume ratio, which confers a compact form on the protein, a reduction in glycine content that tends to remove options for flexibility and thus introduce rigidity to the molecule, and extensive ionic bonding across the protein's surface that helps the compacted protein resist unfolding at high temperature. When thermal stress is applied to any organism the most prominent physiological reactions are the production of a set of novel proteins or

an increase in the quantity of certain types of existing proteins. These proteins can be called as heat shock proteins (HSPs) and they play important roles in the protection of organisms under heat stress [15, 16]. Molecular weight of the protein is major criteria to classify HSPs: According to 1) high molecular size, with molecular size between 39 and 68kDa, medium molecular between 39 and 68 kDa and 3) low molecular size, with molecular weight below 38 kDa. In addition to these some HSPs also called chaperonins are synthesized by hyperthermophiles, which functions to bind with heat denatured proteins and refolds them into their active form.

Thermophiles can ferment similar carbohydrates, utilize similar nitrogen sources, and have similar oxidative pathways. They can exist as aerobes, anaerobes, or as facultative aerobes. There are also autotrophic and heterotrophic species in thermophiles. The search for extremophilic organism is one of the means for obtaining enzymes with properties suitable for industrial purposes (Ibrahim et al., 2007; Turner et al., 2007). Such enzymes have found their way into the grist of industry in applications as diverse as laundry detergent additives (proteases, lipases) and the genetic identification of criminals. DNA polymerases have been obtained from *Thermococcus littoralis*, *Thermus aquaticus*, *Thermotoga maritime*, *Pyrococcus woessii* and *P. furiosus* for application in polymerase chain reaction (PCR). Another important realization that has emerged from the study of extremophiles is that some of these organisms form the cradle of life itself. Many extremophiles, in particular the hyperthermophiles, lie close to the "universal ancestor" of all extant life on Earth. Thus, an understanding of the basic biology of these organisms is an opportunity for biologists to "look backward in time" so

to speak, to a period of early life on Earth. Thermophiles are found in various geothermally heated regions of the earth such as hot springs like those in Yellowstone National Park and deep sea hydrothermal vents, as well as decaying plant matter such as peat bogs and compost. Thermophiles presence can also be seen in compost samples because during composting temperature increase to level where only thermophiles can survive. Composting is the aerobic decomposition of organic materials by microorganisms under controlled conditions into a soil-like substance called compost. During composting, microorganisms such as bacteria and fungi break down complex organic compounds into simpler substances and produce carbon dioxide, water, minerals, and stabilized organic matter (Suler et al., 1977). The composting process at the microbial level involves several interrelated factors, i.e metabolic heat generation, temperature, ventilation, moisture content, and available of nutrients. The temperature both reflects prior microbial activity and current rate of activity. The initial rapid increase of temperature involves a rapid transition from a mesophilic to a thermophilic microflora (Strom, 1985a; Strom, 1985b). The compost ecosystem then tends to limit itself due to inhibitory high temperatures, resulting from excessive heat accumulation (Nakasaki et al., 1985a; Nakasaki et al., 1985b). Aiming isolation of thermophilic microorganism from compost ecosystem will provide a good range of organisms which can survive at the compost temperature (Schulze, 1962).

Materials and Methods

Sample collection

The cattle waste compost samples were collected from (Jaipur, Rajasthan), India. These samples were stored at 60°C and used

for microbiological and analytical study of thermophilic bacteria.

Screening and isolation of thermophilic bacteria

The samples were suspended and diluted serially for 5 times in sterile distilled water. 100µl of each dilution were plated on a nutrient agar plate by using pour plate method and incubated at 60°C for 16-18hr. Morphologically different colonies were picked up, checked for their purity and lyophilized in skim milk. After confirmation, each *Actinobacillus* isolates were maintained on blood agar plates.

Characterization of colonies

Individual colonies were characterized on the basis of colony morphology (shape, size, texture and colour), gram staining and conventional biochemical tests. Single colonies were obtained by using streak plate method. The isolated colony was streaked on nutrient agar slants and blood agar plates and incubated at 60°C for 16hr to obtain optimum growth. The preliminary characterization was based on colony morphology on blood agar plates after 16-18 hr of incubation at 60°C.

Physiological characterization of the isolates

Growth of the isolates was assessed in Nutrient broth at different temperature i.e. 35, 55, 60 and 70°C and different pH 3, 5, 7, 9 and 11 by incubating at 60°C. Salt tolerance was tested by incorporating 1, 2, 3, 5 and 7% (W/V) sodium chloride in NB.

Biochemical Analysis of the bacterial isolates

Individual colonies were characterized by morphological and conventional

biochemical tests like Gram staining, Endospore staining, Motility test, Catalase activity, Starch hydrolysis, Citrate utilization test, Urease test, Oxidase test, Indole test, MRVP test, Nitrate reductase test and Triple sugar iron agar test. These tests were performed according to Microbiology laboratory manual 4th edition by Cappuccino and Sherman (1999). Tentative identification of all bacterial colonies was done with the above tests (Table 1-5).

Extracellular enzyme Production:

Production of extracellular enzyme was determined by incorporation of test substrate into nutrient medium prepared for thermophiles, inoculated the culture to these medium and microorganisms were allowed to grow. After incubation reagents were added to the plates to detect remnant of test substrate. Colony radial growth rate and substrate clearing zone were measured for each organism on each substrate. Three replicates of each treatment were assayed and non inoculated plates with substrate were used as a negative control (Table 1-5).

Amylase- Amylase activity was assayed by growing the fungi on starch medium (Nutrient agar with 2% Starch) and plates were flooded with 1% aqueous solution of potassium iodide. A yellow zone around the colony on a blue back ground was considered as positive test for starch hydrolysis.

Lipase- Lipase activity was determined by growing the isolates on medium (Nutrient agar with 1% Tween 20). Precipitated form of fatty acid crystals can be observed around the colonies in lipase positive microorganisms.

Protease (Proteolytic activity)- Microorganisms were grown on medium

containing gelatin as the protein substrate (Nutrient agar with 0.4% gelatin). After inoculation and proper incubation plates were flooded with an aqueous saturated solution of ammonium sulphate for protein precipitation. Formation of a clear zone around the colonies shows positive test for protease.

After their confirmation as *Actinobacillus*, fermentative activity was evaluated in peptone water (Difco) with Andrade's indicator and each of the following carbohydrates (Difco): mannose, arabinose, xylose, sucrose, maltose, lactose, trehalose, mannitol, and salicin.

Molecular Analysis

Genomic DNA isolation

Genomic DNA was extracted from overnight grown culture in Luria broth. 1.5 ml of culture was taken in fresh autoclaved eppendorf tube and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and pellet was resuspended in 500µl of TAE buffer and vortexed. 10µl of lysozyme (20µg/ml) and 40µl of 10% SDS was added and incubated at 4°C for 15 min. Equal volume of Tris saturated phenol was added and tubes were inverted gently 2-3 times. Centrifugation was done at 12000 rpm for 15 min. The upper aqueous layer, containing DNA was transferred to fresh eppendorf. Equal volume of chloroform: isoamyl alcohol (24:1) was added & mixed by inverting the tubes 4-5 times gently. Tubes were centrifuged at 10000 rpm for 10 min. Upper aqueous layer was taken & transferred to fresh tube and chilled absolute alcohol was added. Tubes were inverted gently 2-3 times & centrifuged at 10000 rpm for 10 min. Supernatant was discarded and pellet was washed with 70% alcohol at 10000 rpm for 10 min. After centrifugation

alcohol was removed completely and pellet was allowed to dry for 1 to 2 hrs. Pellet was dissolved in 50µl sterile TE buffer. The isolated DNA was checked by running them on 0.8% agarose gel with λ DNA marker.

Amplification of 16S rRNA

Amplification of 16S rRNA region was done using PCR with degenerate primers, 25F (5'-AGAGTTTGATCMTGGCTCAG-3') and 514R (5'-GWATTACCGCGGCKGCTG-3') specific to conserved regions. The PCR reaction was done using a BioRad thermal cycler. The volume of reaction mixture was 25µl, containing 8µl of genomic DNA, 2.5µl of 10X PCR buffer (10 mM Tris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM MgCl₂), 1µl of *Taq* DNA polymerase, 1µl of 10mM dNTPs mix and 2µl each of forward and reverse primers (Bangalore Genei, India). The PCR conditions were standardized as follows: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 40s, annealing at 50°C for 45s and primer extension at 72°C for 1min and final extension at 72°C for 7 min. The amplified products were analyzed on a 0.8% agarose gel with ethidium bromide.

Heat Shock proteins profiling

Thermophiles are very well known for heat shock protein expression. Experiment was designed to reveal the heat shock protein expressed by *Actinobacillus*. Bacterial cultures were grown at optimum condition and further incubated at different temperature in water-bath at 50°C, 60°C and 70°C for one hour. Cells were harvested by centrifugation at 12,000rpm for 25 minutes at 20°C and washed twice with sterilized distilled water. Cell pellets were washed thrice with 10mM ice-cold Tris-HCl (pH = 7.6) and then suspended in 0.5ml of 2X SDS sample loading buffer. All samples were

boiled at 100°C for 10 minutes and directly loaded to SDS PAGE.

Results and Discussion

Morphological and biochemical analysis

A total of 20 different morphological forms were isolated from the cattle compost samples collected from Jaipur, India. The ability of these isolates to grow at different temperatures, pH and salt concentrations was studied to find their optimal growth condition. All isolates shows maximum growth at 60°C and isolates Jnu1, Jnu6, Jnu11 and Jnu16 shows growth at 55°C but decrease in temperature effect the growth of culture. Isolate Jnu3 and Jnu13 also shows growth at 70°C but the optimum temperature for all isolate is 60°C (Fig 1).

None of the isolates showed growth at 40°C but all strains had growth at 60°C revealing their thermophilic character and can be classified as moderate thermophiles (Gomes et al., 2004). These bacteria are highly active at 60°C and at temperatures above 60°C the degradation process is performed by these microorganisms (Fogarty et al., 1991; Finstein et al., 1975; Nakasaki et al., 1985a; Strom, 1985b; Sharp et al., 1992). Microbial diversity is a prerequisite for a satisfactory composting process. High temperature more than 60°C are considered to reduce dramatically the functional diversity. It is generally assumed that to obtain an efficient and rapid decomposition, temperature should not be allowed to exceed 55 to 60°C (Mckinley et al., 1984; Turner et al., 2007). Isolates growing at 60°C were further used to study the effect of pH and salt concentration. All the isolates showed maximum growth at pH 7 whereas isolates Jnu5, Jnu7 and Jnu16 showed optimal growth at pH 9 (Fig 2).

Table.1 Biochemical analysis for tentative identification of isolated organism

Isolate	JNU-1	JNU-2	JNU-3	JNU-4	JNU-5
Gram's staining	Gram negative, long rods, bunches.	Gram negative, small rods, highly branched, some also present in chains	Gram negative, small rods, bunches & chain	Gram negative, very long rods, branched.	Gram negative, rods, branched, filamentous, spores present
Endospore staining	-	-	-	-	-
Motility	-	-	-	-	-
Catalase	+	+ but delayed	+	+	+
Starch hydrolysis	+	+	+	+	+
Protease	+	+	+	+	+
Lipase	+	+	+	+	+
Citrate	-	-	-	-	-
Urease	-	-	-	-	-
Oxidase	+	+	+	-	+
Indole	+	+	+	+	+
Lysozyme	+	+	+	+	+
Methyl red	+	-	-	-	-
Voges Proskauer	-	+	+	+	+
Nitrate reductase	Without Zn	+	+	+	+
	With Zn	-	-	-	-
Oxidative – fermentative test	Glucose	+	+	+	+
	Lactose	+	+	+	+
	Sucrose	-	+	-	+
	Maltose	+	+	+	+
	Fructose	+	+	+	+
Triple Sugar Iron Agar	-	-	-	-	-
Tyrosine	+	+	-	+	-
Tentative identification	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>
Similarity index	0.73596	0.87654	0.95866	0.75467	0.76754

Table.2 Biochemical analyses for tentative identification of isolated organism

Isolate	JNU-6	JNU-7	JNU-8	JNU-9	JNU-10
Gram's staining	Gram negative, long rods, bunches.	Gram negative, small rods, highly branched, some also present in chains	Gram negative, small rods, bunches & chain	Gram negative, very long rods branched.	Gram negative, rods, branched, filamentous, segmented, spores present.
Endospore staining	-	-	-	-	-
Motility	-	-	-	-	-
Catalase	+	+ but delayed	+	+	+
Starch hydrolysis	+	+	+	+	+
Protease	+	+	+	+	+
Lipase	+	+	+	+	+
Citrate	-	-	-	-	-
Urease	-	-	-	-	-
Oxidase	+	+	+	-	+
Indole	+	+	+	+	+
Lysozyme	+	+	+	+	+
Methyl red	+	-	-	-	-
Voges Proskauer	-	+	+	+	+
Nitrate reductase	Without Zn	+	+	+	+
	With Zn	-	-	-	-
Oxidative – fermentative test	Glucose	+	+	+	+
	Lactose	-	+	-	+
	Sucrose	+	+	+	+
	Maltose	-	-	-	-
	Fructose	+	+	+	+
Triple Sugar Iron Agar	-	-	-	-	-
Tyrosine	-	+	+	-	-
Tentative identification	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>
Similarity index	0.97129	0.95467	0.78912	0.75321	0.90122

Table.3 Biochemical analyses for tentative identification of isolated organism

Isolate	JNU-11	JNU-12	JNU-13	JNU-14	JNU-15	
Gram's staining	Gram negative, long rods, bunches.	Gram negative, small rods, highly branched, some also present in chains	Gram negative, small rods, bunches & chain	Gram negative, very long rods, branched.	Gram negative, rods, branched, filamentous, segmented, spores present.	
Endospore staining	-	-	-	-	-	
Motility	-	-	-	-	-	
Catalase	+	+	+	+	+	
Starch hydrolysis	+	+	+	+	+	
Protease	+	+	+	-	-	
Lipase	+	+	+	-	-	
Citrate	-	-	-	-	-	
Urease	-	-	-	-	-	
Oxidase	+	-	-	+	-	
Indole	+	+	+	+	+	
Lysozyme	+	+	+	+	+	
Methyl red	+	+	+	-	-	
Voges Proskauer	-	-	-	+	+	
Nitrate reductase	Without Zn	+	+	+	-	
	With Zn	-	-	-	+	
Oxidative – fermentative test	Glucose	+	+	+	+	
	Lactose	+	+	+	+	
	Sucrose	-	+	-	+	-
	Maltose	-	-	-	-	-
	Fructose	+	+	+	+	+
Triple Sugar Iron Agar	-	-	-	-	-	
Tyrosine	-	-	-	+	+	
Tentative identification	<i>Actinobacillus lignieressi</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus</i>	<i>Actinobacillus lignieressi</i>	
Similarity index	0.73694	0.86143	0.67120	0.86432	0.75467	

Table.4 Biochemical analyses for tentative identification of isolated organism

Isolate	JNU-16	JNU-17	JNU-18	JNU-19	JNU-20	
Gram's staining	Gram negative, short rods, single & bunches	Gram positive, long rods, single & group	Gram negative, long rods, single & group	Gram positive, small rods, groups.	Gram negative, small rods, bunch	
Endospore staining	-	-	-	-	-	
Motility	-	-	-	-	-	
Catalase	+	+	+	+	+	
Starch hydrolysis	+	+	+	+	+	
Protease	+	+	+	+	+	
Lipase	-	+	+	-	+	
Citrate	-	-	-	-	-	
Urease	-	-	-	-	-	
Oxidase	-	+	+	+	-	
Indole	+	+	+	+	+	
Lysozyme	+	+	+	+	+	
Methyl red	-	-	-	-	-	
Voges Proskauer	+	+	+	+	+	
Nitrate reductase	Without Zn	+	-	-	+	
	With Zn	-	-	+	-	
Oxidative – fermentative test	Glucose	+	+	+	+	
	Lactose	+	+	+	+	
	Sucrose	+	+	+	+	
	Maltose	-	-	-	-	-
	Fructose	+	+	+	+	+
Triple Sugar Iron Agar	-	-	-	-	-	
Tyrosine	+	+	-	-	-	
Tentative identification	<i>Actinobacillus lignieressi</i>	Not identified	Not identified	Not identified	<i>Actinobacillus lignieressi</i>	
Similarity index	0.73694				0.94647	

Table.5 Fermentative reaction of *Actinobacillus lignieressii*

Carbohydrate	24 hrs	48hrs
Glucose	+	+
Xylose	+	+
Mannitol	+	+
Lactose	-	-
Sucrose	+	+
Maltose	+	+
Trehalose	-	-
Salicin	-	-
Arabinose	+	+

In the composting process, initially pH goes down because of acid production and then increases by ammonia production. So the isolates are adapted to neutral pH. Four isolates (Jnu4, Jnu9, Jnu17 and Jnu20) showed optimum growth at 1% salt concentration. All isolates except Jnu3 and Jnu5 were able to grow at 2% NaCl concentration compared to higher salt concentration. Isolate Jnu14 was unable to tolerate even 1% salt concentration. Isolate Jnu8 and Jnu17 showed growth up to 7% salt concentration (Fig 3).

These isolates were identified on the basis of morphological, physiological & biochemical characteristics using software for probabilistic identification of bacteria (<http://www.som.soton.ac.uk/staff/tnb/pib.html>). Colonies were non haemolytic, small, raised, and greyish white. Majority of organisms stained as pleomorphic gram-negative rods. Out of 20 isolates 90% were gram negative and the rest were found to be gram positive. Spore formation was not observed for any of the isolates (Table: 1-4). Heat resistant spores were found among several species, mainly those belonging to the genera *Bacillus* and *Clostridium* (Fernández et al., 2001; Hyun et al., 1983). Spore formation was not observed in the case of *Actinobacillus*. In previous research also less attention has been paid to thermophilic heat resistant spores than mesophilic spores which are used in food industry.

Spores from thermophilic bacteria are more heat resistant than spores from mesophilic species (Warth, 1978). Assay was performed to check the extracellular enzyme production by these isolates and most of the isolates found to be positive for protease, lipase and amylase activity. These 20 isolates were identified to belong to *Actinobacillus* genera and out of these 4 isolates identified to species level as *Actinobacillus lignieressi* by using the software (<http://www.som.soton.ac.uk/staff/tnb/pib.htm>). Isolates which were regarded as a possible *Actinobacillus* after initial biochemical tests were further evaluated for fermentative activity. The isolates were inoculated into peptone water with Andrade's indicator and each of the following carbohydrates: mannose, arabinose, xylose, sucrose, maltose, lactose, trehalose, mannitol, and salicin. We used the differential identification scheme because *Actinobacillus* identification is very confusing. Thermophilic nature and ability to secrete extracellular enzyme of these isolates indicates their possible role in various industries.

16S rRNA region amplification and sequencing

The DNA was isolated from 10 isolates of *Actinobacillus* showing optimum growth at 60°C which indicates their non pathogenic nature. The 16S rRNA region of 600bp

amplified from these isolates by using 514R as a reverse primer and 25F as a forward primer (Fig 4).

Sequencing has been done and compared with the sequence already submitted to Genebank of National Central for Biotechnology. Nucleotide sequence data reported in this paper are available in the Genebank, EMBL and DDBJ databases under the accession number: JQ783352

Heat Shock proteins profiling

Thermophiles are eminent source for heat shock protein production. Study has been done to find the heat shock protein expressed by *Actinobacillus*. In this study both high molecular weight and low molecular weight heat shock proteins were expressed by *Actinobacillus*. The molecular weight of heat shock proteins ranged from 18.4 to 100 KDa.

Study was initiated to study the presence of thermophiles in compost samples but after experimental analysis we found that *Actinobacillus* which generally survive in moderate temperature can sustain higher temperature also. It has been found that *Actinobacillus* was the most dominant bacteria in cattle compost. *A. lignieresii* is responsible for actinobacillosis, an infectious, chronic, generally non-fatal disease (Rebhun et al., 1988). In cattle, it typically infects the tongue and hence is also known as “the Wooden Tongue” (Misra et al., 1981; Mohanty et al., 1970). The involvement of other organs is considered to be atypical (Aslani et al., 1995; Holzhauser et al., 2002; Rebhun et al., 1988; Mallick et al., 1984; Misra et al., 1981). The etiologic agent is found normally in the oropharynx and rumen of cattle and sheep (Smith, 1990; Songer et al., 2005). The organism is an opportunistic pathogen and it causes chronic

pyogranulomatous lesions of the soft tissues of head and neck regions in cows, buffalo, sheep, goats, and horses (Baum et al., 1984; Fubini et al., 1983; Hirsh et al., 2004; Kumar et al., 1998; Songer et al., 2005). Clinically, the lesions appear as nodules, multiple abscesses, ulcers or draining fistulae. *A. lignieresii* is causative agent for spontaneous actinobacillosis in the buffalo (*Bubalus bubalis*) which reported as non-fatal (Ahmad et al., 1986; Akhtar et al., 1964). In previous studies *A. lignieresii* is already described as causative agent of a granulomatous disease of cattle and it was also responsible for the prevalent form of the similar. Report shows that *Actinobacillus* can also cause infection in human which escort to death (Beaver et al., 1933) previous report on *Actinobacillus* submitted by our group also shows its presence in cattle compost. Isolation of *Actinobacillus* has been already done from laboratory rodents, (Lentsch et al., 1980; Simpson et al., 1980) but characterization of these organisms is still mystery; host range for the organism is still incomplete. Optimum growth condition for *Actinobacillus* is 37°C temperature and acidic pH but in this study we found that it shows adaptation for 60°C temperature also. It was found that increase in temperature can change the protein expression level of the organisms and may be these proteins are responsible for their survival on high temperature. *Actinobacillus* also shows degradation ability in these high temperature conditions. Amino acid composition of surface proteins in mesophylic and thermophilic bacteria is different, which is responsible for the survival in harsh condition, which shows that different set of proteins, are expressed in these conditions. Protein expression and different metabolic activity of *Actinobacillus* in these conditions was studied and proteins were isolated from *Actinobacillus* and molecular mass was determined. Peptide mass fingerprinting is in

process for further characterization of these proteins. These days' researchers are more interested in proteins which are expressed in those organisms which helps them to sustain a particular kind of environment. Till date there is no report for protein expression studies on *Actinobacillus spp.* According to our knowledge this is the first report of *Actinobacillus* from India in which one pathogenic organism got adapted to thermophilic nature and lost its

pathogenicity. This study also generates several questions in our mind like; how a mesophilic pathogenic organism converted to non pathogenic thermophilic organism. Expression of different set of gene is required to sustain that environment and what were the factors which functions as an initiator. Another big question is how organism lost its pathogenicity after acquiring that adaptation.

Fig.1 Effect of temperature on bacterial isolates.

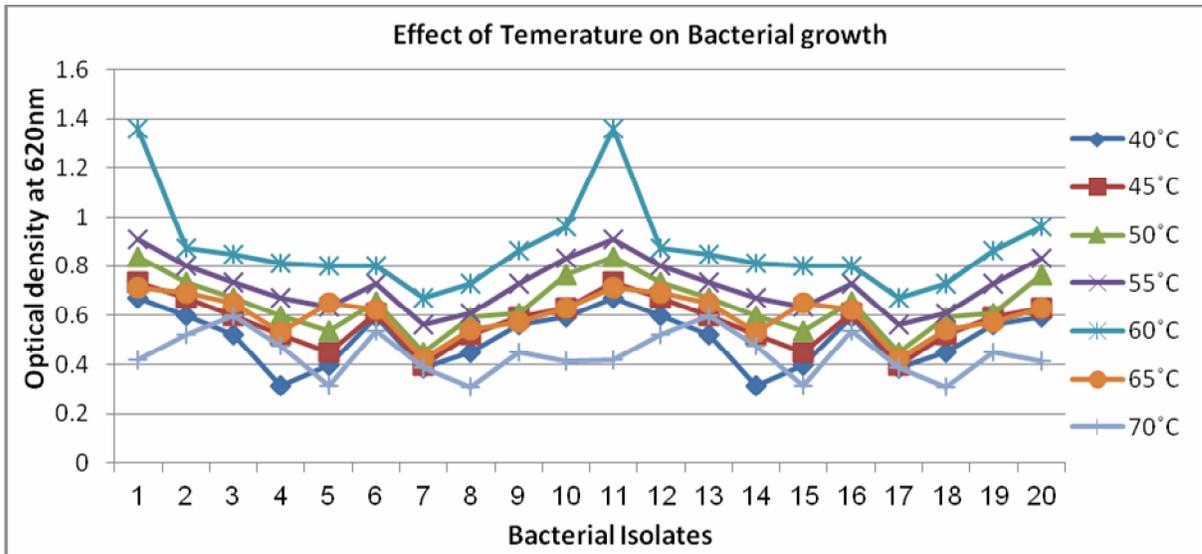


Fig.2 Effect of pH on bacterial isolates.

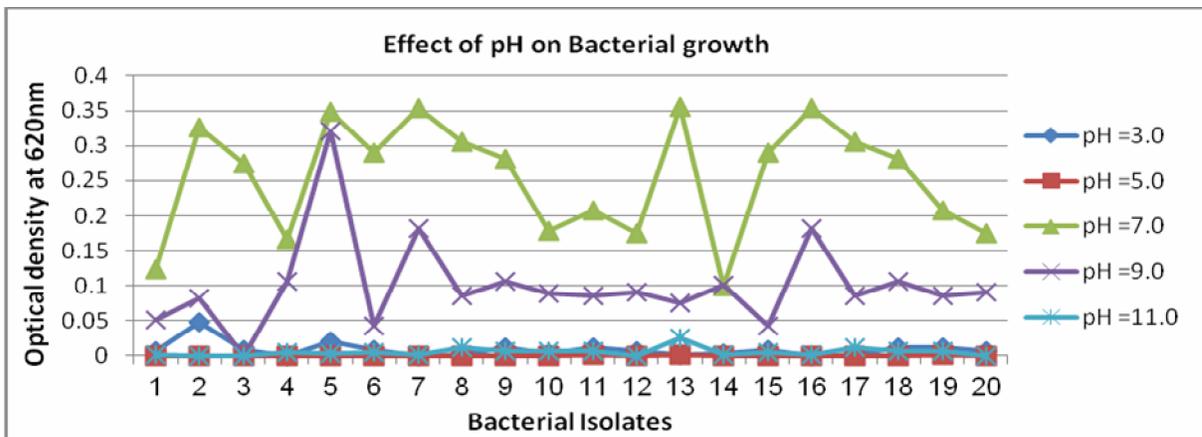


Fig.3 Effect of salt concentration on bacterial isolates.

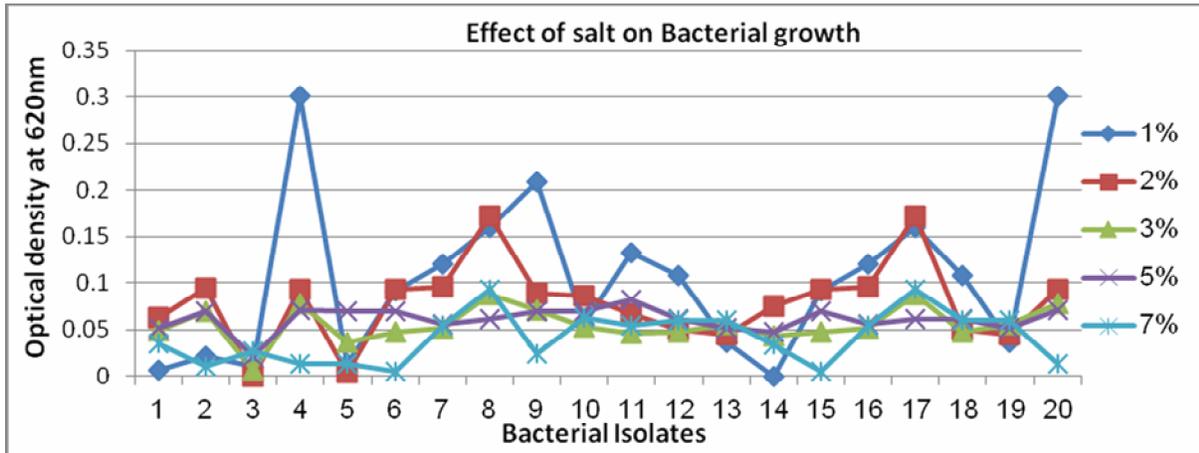


Fig.4 DNA isolated from *Actinobacillus* isolates

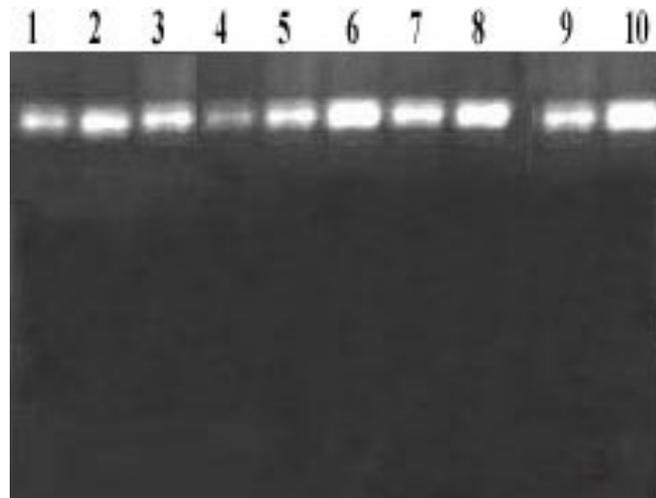


Fig.5 *A. lignieresii* DNA amplified by 514R and 25F primer.

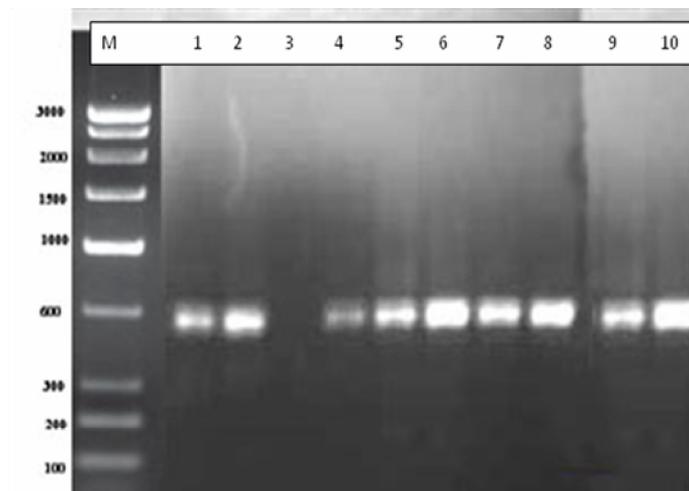


Fig.6 Effect of heat shock treatment on the pattern of protein synthesis in *Actinobacillus*. Bacterial cultures was placed in 40°C (control), 60°C(control) , 50°C, 60°C and 70°C (heat shock) water bath for 1 hour. The position of standard protein markers and the size of the heat shock proteins are marked. [Lane 1 = Low range protein marker, Lane 2 = 40°C, Lane 3 = 60°C, Lane 4 = 50°C (heat shock), Lane 5 = 60°C (heat shock), Lane 6 = 70°C (heat shock)]

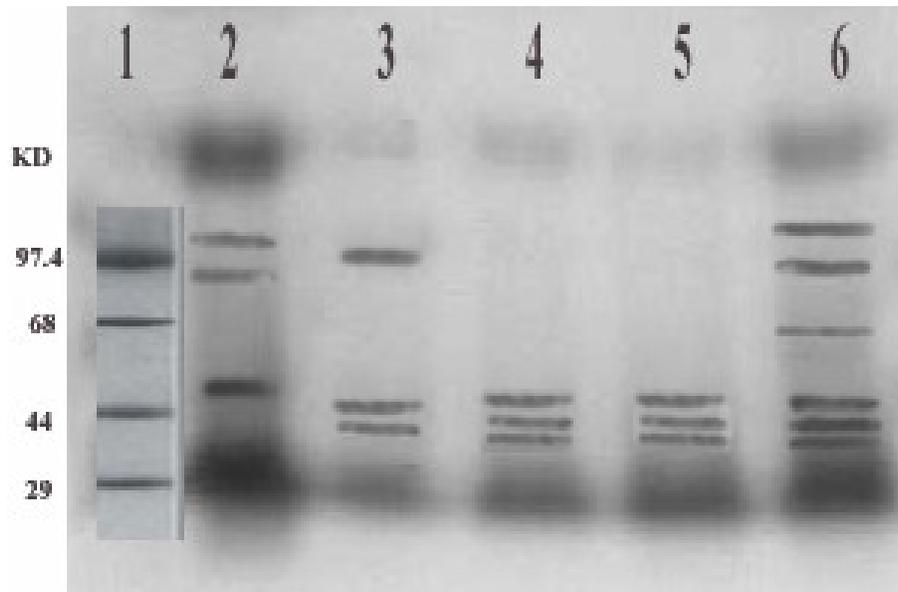
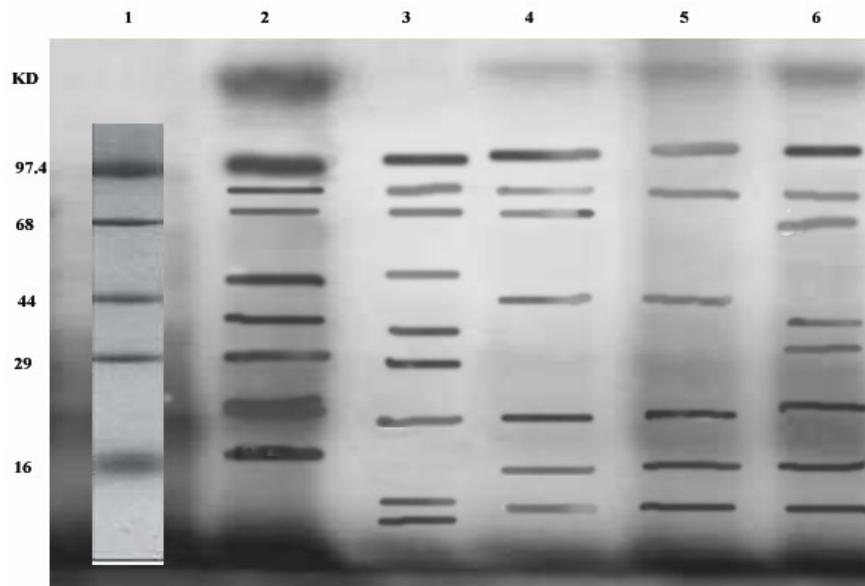


Fig.7 Effect of heat shock treatment on the pattern of protein synthesis in *Actinobacillus*. Bacterial cultures was placed in 40°C (control), 60°C(control) , 50°C, 60°C and 70°C (heat shock) water bath for 2 hour. The position of standard protein markers and the size of the heat shock proteins are marked. [Lane 1 = Low range protein marker, Lane 2 = 40°C, Lane 3 = 60°C, Lane 4 = 50°C (heat shock), Lane 5 = 60°C (heat shock), Lane 6 = 70°C (heat shock)]



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