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STUDY ON THERMOPHILES REVEALS THE PRESENCE OF *ACTINOBACILLUS LIGNIERESII* IN CATTLE COMPOST

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Abstract

Introduction- Moderate environment is important to sustain life any environment condition that can be perceived as beyond the normal acceptable range is extreme condition and if we are talking in the terms of temperature than its called thermophillic environment. **Aim & objective-** The study was designed to study the diversity of thermophiles present in compost and characterization of the properties which are responsible for their adequate nature to sustain high temperature. **Material and method-** 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. **Results-** Isolates from compost showed wide range of growth temperatures which suggest their important activity during the whole composting process. This study initially designed to study the diversity of thermophiles in compost which turned specifically to *A. lignieresii*.

Key words- compost, thermophiles, *Actinobacillus*, biochemical, molecular analysis.

Introduction

Moderate environment is important to sustain life. Moderate means environments with near neutral pH, temperature between 20 and 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. This type of organisms is 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. In addition to these intrinsic stability factors, special proteins 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 woesii* 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).

Table 1: Biochemical analysis for tentative identification of isolated organism

Isolate		A1	A3	A4	A5	A6	A7	A8	A9
Gram's staining		Gram negative, long rods, bunches, endospore forming	Gram negative, small rods, bunches	Gram negative, small rods, bunches & chain	Gram positive, long rods, bunches, endospore forming	Gram negative, rods, bunches	Gram positive, long rods, single, endospore forming	Gram negative, short rods, single & bunches, endospore forming	Gram negative, rods, single & chain endospore forming
Endospore staining		+	-	-	+	-	+	+	+
Motility		-	-	-	+	-	+	+	-
Catalase		+	-	-	-	+	+	+	+
Starch hydrolysis		-	-	-	+	+	+	-	+
Citrate		-	-	-	-	-	-	-	-
Urease		-	-	+	-	-	-	-	-
Oxidase		+	+	-	-	+	+	-	-
Indole		-	-	-	+	-	-	-	-
Methyl red		-	-	-	-	-	-	-	-
Voges Proskauer		-	-	-	-	-	-	-	-
Nitrate reductase	Without Zn	+	-	-	-	-	+	-	+
	With Zn		+	+	+	+		+	
Triple sugar iron agar	Glucose	-	+	+	-	-			-
	Lactose	-	+	-	-	-			-
	Sucrose	-	+	-	-	-			-
Tentative identification		*Brahnamella*	*Actinobacillus lignieressi*	*Pasteurella ureae*	*Streptococcus cremoris*	*Pasteurella haemolytica*	*Micrococcus*	*Erwinia herbicola*	*Bordetella*
Similarity index		0.93193	0.28138	0.44953		0.74153	0.58468	0.52585	0.786

Table 2: Biochemical analyses for tentative identification of isolated organism

Isolate		A10	A11	A13	A14	A15	A17	A18	A19
Gram's staining		Gram negative, short rods, single & bunches	Gram positive, long rods, single & group	Gram positive, long rods, single & group	Gram negative, small rods, groups, endospore forming	Gram negative, small rods, bunch	Gram positive, long rods, endospore forming	Gram negative, small rods, bunch	Gram positive, single rods, endospore forming
Endospore staining		-	-	-	+	-	+	-	+
Motility		-	-	+	-	-	+	+	+
Catalase		-	+	-	+	+	+	-	-
Starch hydrolysis		-	+	+	+	-	-	-	-
Citrate		-	-	-	-	-	-	-	-
Urease		-	-	-	-	-	+	+	-
Oxidase		+	+	-	+	+	-	-	-
Indole		-	-	+	-	-	-	-	-
Methyl red		-	-	-	-	-	-	-	-
Voges Proskauer		-	-	-	-	-	-	-	-
Nitrate reductase	Without Zn	-	+	+	+	-	-	-	-
	With Zn	+				+	-	+	+
Triple sugar iron agar	Glucose	+	-	-	-	-	-	+	-
	Lactose	+	-	-	-	+	-	+	-
	Sucrose	+	-	-	-	+	-	+	-
Tentative identification		*Actinobacillus lignieressi*	Not identified	Not identified	*Pasturella haemolytica*	*Pasturella haemolytica*	Not identified	*Actinobacillus lignieressi*	*Micrococcus*
Similarity index		0.73694			0.40920	0.39572		0.94647	

Table 3: Biochemical analyses for tentative identification of isolated organism

Isolate		A20	A21	A22	A23	A25	A26	A28	A29
Gram's staining		Gram positive bacilli, single & bunches	Gram negative, small rods, bunches	Gram negative, small rods, bunches	Gram positive, short rods, chains, endospore forming	Gram negative, short rods, bunches	Gram negative, short rods, bunches	Gram negative, small rods, bunches	Gram positive, small rods, bunches
Endospore staining		-	-	-	+	-	-	-	-
Motility		+	-	-	+	+	-	-	-
Catalase		-	-	+	+	-	-	-	-
Starch hydrolysis		-	-	-	+	-	-	-	-
Citrate		+	-	-	-	-	-	-	+
Urease		-	+	+	-	-	+	-	+
Oxidase		+	+	+	-	-	+	+	+
Indole		-	-	-	-	-	-	-	-
Methyl red		-	-	-	-	-	-	-	-
Voges Proskauer		-	-	-	-	-	-	-	-
Nitrate reductase	Without Zn	-	-	-	+	-	-	-	-
	With Zn	+	+	+		+	+	+	+
Triple sugar iron agar	Glucose	-	+	-	-	-	+	-	+
	Lactose	+	+	+	-	+	+	-	+
	Sucrose	+	+	+	-	+	+	-	+
Tentative identification		Not identified	*Actinobacillus lignieresii*	*Pasturella ureae*	Not identified	*Erwinia herbicola*	*Actinobacillus lignieresii*	*Kingella kingae*	*Actinobacillus lignieresii*
Similarity index			0.75512	0.70177		0.41934	0.99086	0.74427	0.75467

Table 4: Biochemical analyses for tentative identification of isolated organism

Isolate		A30	A31	A32	A33	A34	A35
Gram's staining		Gram positive, small rods, bunches	NA	Gram negative, single, small rods, bacilli	Gram positive, small rods, bunches	Gram negative, rods, bunches	Gram negative, short rods, bunches & endospore forming
Endospore staining		-	-	-	-	-	+
Motility		+	-	-	-	-	-
Catalase		+	+	-	--	-	-
Starch hydrolysis		-	-	-	-	-	+
Citrate		-	-	-	-	-	-
Urease		-	+	+	-	-	+
Oxidase		+	+	+	-	+	+
Indole		+	-	-	-	-	-
Methyl red		-	-	-	-	-	-
Voges Proskauer		-	-	-	-	-	-
Nitrate reductase	Without Zn	-	-	-	-	-	-
	With Zn	+	+	+	+	+	+
Triple sugar iron agar	Glucose	-	-	+	-	-	+
	Lactose	+	+	+	-	-	+
	Sucrose	+	+	+	-	-	+
Tentative identification		Not identified	*Agrobacterium*	*Actinobacillus lignieresii*	Not identified	*Kingella kingae*	*Actinobacillus lignieresii*
Similarity index			0.52365	0.55006		0.71915	0.50123

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

Table 5: Fermentative reaction of *Actinobacillus lignieresii*

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

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 4^0C 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, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5-' GWATTACCGCGGCKGCTG-3') specific to conserved regions. The PCR reaction was done using a BioRad thermal cycler. The volume of reaction mixture was 25 µl, containing 8µl of genomic DNA, 2.5µl of 10X PCR buffer (10 mM Tris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 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.

Results

Morphological and biochemical analysis

A total of 30 different morphological forms were isolated from the cattle waste compost 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. The isolates A3, A4, A10, A15, A18, A20, A21, A25, A26, A29, A30, A31, A32 and A35 showed maximum growth at 55°C and the isolates A1, A5, A6, A7, A8, A9, A11, A13, A14, A17, A19, A23, A33 and A34 at 60°C (Fig.1).

The optimum temperature of growth is 70°C for the isolates A20, A30 and A32. None of the isolates showed growth at 35°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). Of the 30 isolates, 14 having good growth at 55 and 60°C were further used to study the effect of pH and salt concentration. All the isolates showed optimal

growth at pH 7 whereas isolates A15 and A20 showed optimal growth at pH 9 (Fig. 2).

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 (A10, A25, A29 and A30) showed optimum growth at 1% salt concentration. All isolates were able to grow at 2% NaCl concentration compared to higher salt concentration. Isolate A9 was unable to tolerate even 1% salt concentration. Isolate A21 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.htm).
Colonies were non haemolytic, small, raised, and greyish white. Majority of organisms stained as pleomorphic gram-negative rods. Out of 30 isolates 66.6% were gram negative and the rest were found to be gram positive. Spore formation was observed in 5 gram positive isolates (A5, A7, A17, A19 and A23) and 5 gram negative isolates (A1, A8, A9, A14, A35) (Table: 1-4). Heat resistant spores were found among several species, mainly those belonging to the genera *Bacillus* and *Clostridium* (Ferna´ndeza et al., 2001; Hyun et al., 1983). 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). Protease and cellulases activity is not there in any of the isolates. These 30 isolates were identified to belong to 8 genera namely *Actinobacillus, Pasteurella, Micrococcus, Brahnamella, Erwinia, Kingella, Bordetella* and *Agrobacterium.* Of these 18 isolates were identified up to species level using the software (http://www.som.soton.ac.uk/staff/tnb/pib.htm). 8 isolates were identified as *A. lignieresii*, 3 isolates as *Pasteurella haemolytica,* 2 isolates as *Pasteurella ureae,* 2 isolates as *Erwinia herbicola,* 2 isolates as *Kingella kingae* and one isolate as *Streptococcus cremoris.* 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.

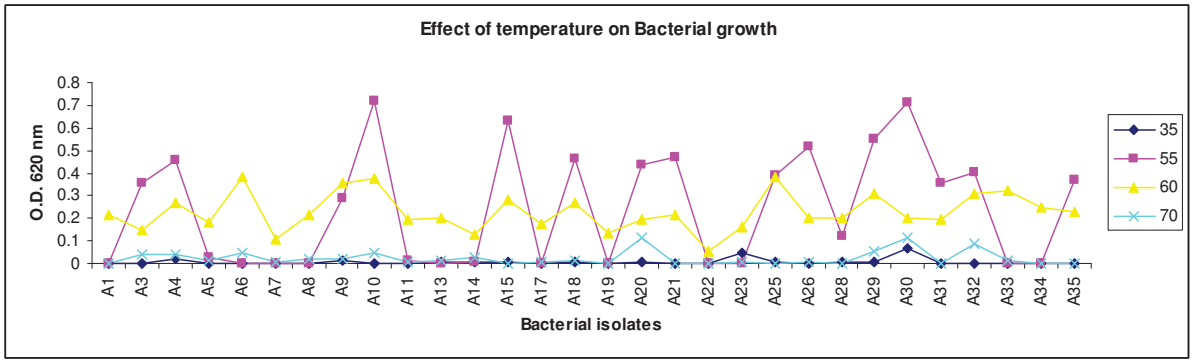

Fig. 1: Effect of temperature on bacterial isolates.

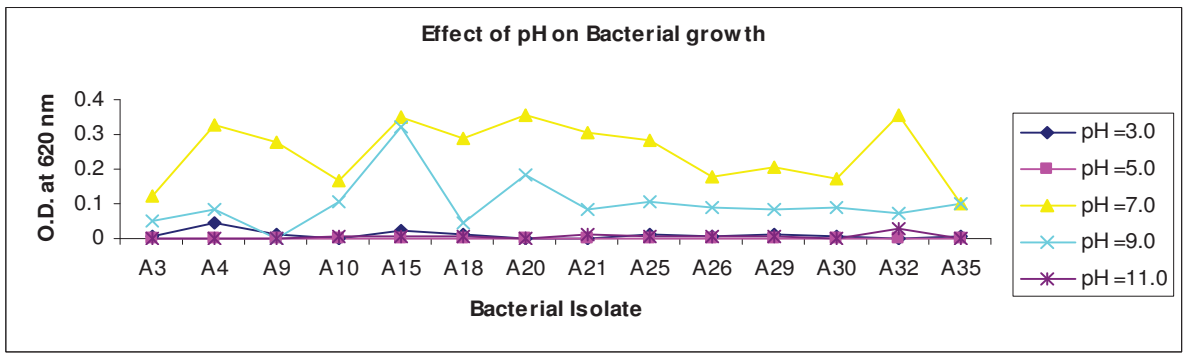

Fig. 2: Effect of pH on bacterial isolates.

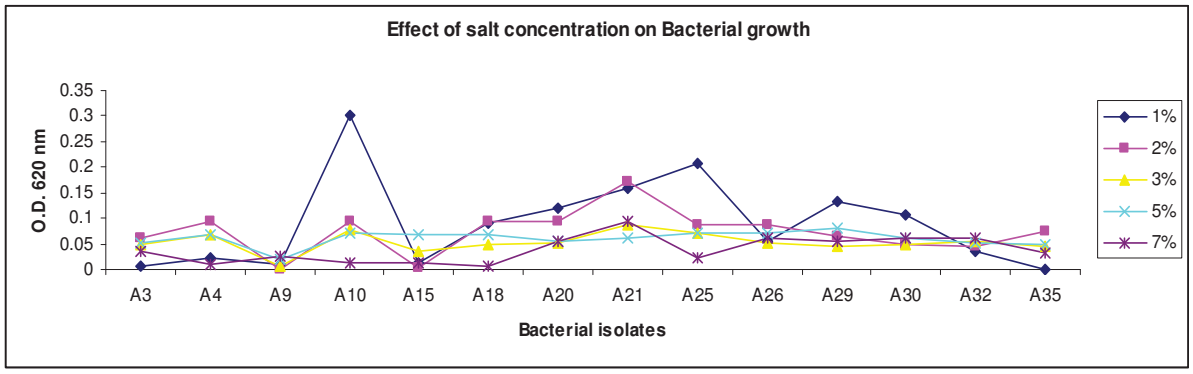

Fig. 3: Effect of salt concentration on bacterial isolates.

16S rRNA region amplification and sequencing

The DNA was isolated from these eight isolates of *Actinobacillus* which were unable to grow below 55°C indicating their nonpathogenic nature. The 16S rRNA region of 991bp amplified from these isolates by using 512R as a reverse primer and 27F as a forward primer (Fig 4).

Sequencing has been done and submitted, for the amplified region 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

Discussion

The study was designed to study thermophiles 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; Holzhauer 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) but no recent reports are available for that.

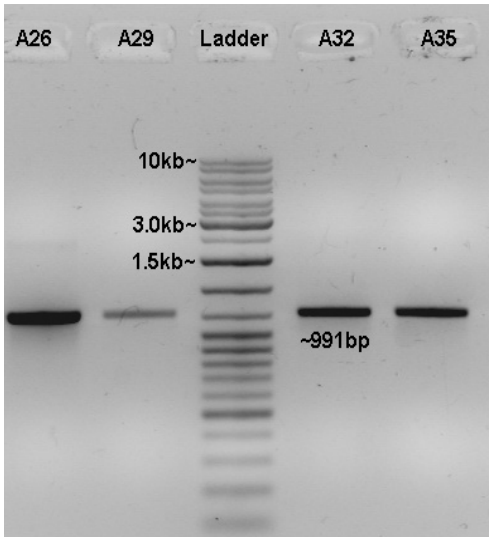

Fig. 4: *A. lignieresii* DNA amplified by 519R and 27F primer

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. Justification can be given as that during composting process temperature rises 60 to 70°C and *Actinobacillus* make itself to survive in those conditions. *Actinobacillus* also shows degradation ability in these high temperature conditions. Amino acid composition of surface proteins in mesophylic and thermophilc bacteria is different, which 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 is yet to

be studied. These days' researchers are more interested in proteins which are expressed in those organisms which helps them to sustain that 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 pathogenecity. 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 pathogenecity after acquiring that adaptation.

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