Advances in Life Science and its Applications (ALSA)
Vol. 1, No. 3, 2012

www.worldsciencepublisher.org

BIOCHEMICAL CHARACTERISTICS OF A POLYCYCLIC AROMATIC HYDROCARBON DEGRADING BACTERIUM ISOLATED FROM AN OIL REFINERY SITE OF WEST BENGAL, INDIA.

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Abstract – Polycyclic aromatic hydrocarbons are a class of potentially hazardous chemicals. Their contamination of soil and aquifers is of great environmental concern today as humans throughout the world may be exposed to these compounds from different sources. Due to high boiling point and chemical stability they can not be easily removed from the environment either by evaporation or chemical reactions. They are very much toxic; some of them mutagenic, even carcinogenic. Some microorganisms have been able to transform these toxic chemicals to nontoxic ones (bioremediation). The objective of this study is to isolate any potent bacteria from Haldia Refinery site of India that may degrade multiple polycyclic aromatic hydrocarbons (PAHs). An isolate has been identified as *Bacillus weihenstephansis* DSM 11821 and characterized with properties like multiple PAH degradation, biosurfactant, lipase and gelatinase activity.

Keywords – Bioremediation; biosurfactant; lipase; petroleum oil; polycyclic aromatic hydrocarbons

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are fused-ring (with more than one aromatic ring) hydrocarbon compounds produced from incomplete combustion of organic materials either from natural sources as forest fires and volcanic eruptions or from anthropogenic sources like petroleum refining plants, fossil fuel burning, plastic waste incineration. Sometimes oil spillage (during transportation), pipe leakage and also accidents by oil tankers result the environmental pollution by the PAHs. Haldia Refinery (a major petroleum oil refinery unit of Indian Oil Corporation Limited, ranked 98th by the Fortune 500 for the year 2011) has been in operation since 1975 and so the environment of this region is highly polluted with the release of different PAHs. They enter our body either through the food chain (6, 32) or when we expose to them in the air.

United States Environmental Protection Agency (USEPA) has identified the 16 PAHs as priority pollutants (22), even the low molecular weight PAH have some mild effect but that could be potentially hazardous for occupational people (19, 23, 34). Phenanthrene is known to be a human skin photosensitizer and mild allergen (11). Many other PAHs are much toxic, even mutagenic (15, 18, 20). Some PAHs have been even tested for carcinogenicity in various animals (44) and resulted in both benign and malign tumors (28). They cause different type of cancer (36, 42), depressed immune function (14). They also have deleterious effects on newborn babies (12). Since PAHs are highly recalcitrant under normal conditions due to their structural complexity and strong molecular bonds (37), they have relative resistance to chemical or photooxidation. So they are difficult to remove from the environment. Thus there has been increasing interest in the bioremediation of terrestrial and aquatic PAH-contaminated environments. Bioremediation is a process by which contaminated regions are restored by means of biogeochemical process. Interest in bioremediation is prompted by their ubiquitous distribution and the potential self-cleaning process of the indigenous microorganisms (3, 39, 40).

2. Materials and Methods

Soil collection and determination of pH. Soil was collected from 1 ft depth of the selected site. 5 gm dry soil was dissolved in 25 ml 0.5mM $CaCl_2$ (pH-7.0) solution and then measured its pH with pH meter (30).

Isolation of a multiple PAH degrader

A. Anthracene and benzo(a)pyrene degrading bacteria. A mineral salt medium (MSM) was prepared (1) with some modifications of NH_4Cl 2.0gm, KH_2PO_4 5.0gm, Na_2HPO_4 4.0gm, $MnSO_4$ 0.2 gm, $MgSO_4$ 0.2gm, $FeCl_3$ 0.05gm, $CaCl_2$ 0.001gm, $CoCl_2$ 0.001gm, $(NH_4)_6Mo_7O_2$ 0.0001 gm per lit, pH maintained at 7.2 (pH of the soil). The medium was filtered through Whatman filter paper (No.-1) and then autoclaved. One

gm soil was suspended in 10 ml MSM medium, mixed thoroughly, centrifuged at 1000 rpm for 10 mins, and allowed to stand for 2 hr. Supernatant was collected and centrifuged at 10000 rpm for 10 mins. Pellet was washed with MSM medium twice and then resuspended in 5ml. MSM medium. 1 ml of this bacterial suspension was separately added to three conical flasks, each containing 200 ml MSM medium, one supplemented with solid crystals of anthracene, one benzo(a)pyrene (Sigma) as sole source of carbon and another containing no carbon source, then incubated at 30^0C, 100 rpm. After 10 days 1 ml of suspension was taken from each flask and the whole process repeated twice. After that they were plated on three different nutrient agar plates.

B. Phenanthrene degrading bacteria. A set of 8 test tubes were taken that contain (filter sterile) 20 mg phenanthrene dissolved in acetone, then acetone was removed by evaporation. To each of the different tubes were added 20 ml MSM medium and PAH degrading bacteria (10^6 cells) isolated form eight different colonies on nutrient agar plate, then incubated at 30^0C, 100 rpm for 10 days. Bacterial growth was measured in terms of optical density at 600 nm by UV spectrophotometer (Simadzu).

C. Degradation of fluoranthene. Some isolates were inoculated on MSM agar plate and then acetone solution of fluoranthene was sprayed over the plates (17), acetone was vaporized and the plates were incubated at 30^0C for 7 days.

Growth curve on fluoranthene. The best isolate (about 10^7) was inoculated to 50 ppm fluoranthene (sole source of carbon) containing MSM medium, and then incubated at 30^0C, 100 rpm for 7 days.

Biochemical characterization. Gram stain, motility test and other common biochemical tests were performed (5).

Gelatin zymogram. Isolate was cultured in MSM medium supplemented with 2% hexadecane for 3 day. Then bacterial extract was run on 12% SDS PAGE supplemented with gelatin (2%) as substrate about 90 mins, using Coomassie Brilliant Blue R-250 as the staining solution.

Lipase and lecithinase activity. Egg yolk agar plate was prepared with a composition (29) of peptic digest of animal tissue 40g, dextrose 2g, disodium phosphate 5g, monosodium phosphate 1g, NaCl $_2$g, $MgSO_4$ 0.1g, agar 25g, egg yolk 100 ml, pH-7.2, Bacteria was streaked on the plate and then incubated at 30^0C for 48 hrs.

Hemolytic activity. Hemolytic activity of the isolate was determined by streaking the culture on sheep blood (10% V/V) agar plate at 30^0C for 48 hrs (33).

Measurement of surface tension. 25 ml of the culture was collected after 1, 2, 3 and 4 days of incubation followed by centrifugation at 9000 rpm for 15 min at 4^0 C. Surface tension (ST) of the supernatant was measured by drop count method using stalagmometer with the following equation (10)

$$\sigma_L = \frac{\sigma_w N_w \rho_L}{N_L \rho_w}$$

where ρ_L is the surface tension of the liquid under investigation, $\int_W$ is the surface tension of water (72 dyne/cm), N_L is the number of drops of the liquid, N_W is the number of drops of water, $\rangle_L$ is the density of the liquid and $\rangle_W$ is the density of water.

Emulsification activity. 1 ml cell free extract (after 4 day of incubation) was mixed with 4 ml kerosene in a test tube, then vortex at high speed for 2 mins, allowed standing for 1 hr.

Nature of biosurfactant. To understand the nature of the biosurfactant (anionic type like glycolipid or cationic type like lipopeptide) CTAB-Methylene blue test and paper chromatography were done respectively. In one set Cetyltrimethylammonium bromide (CTAB)-methylene blue agar was prepared by adding 0.2 g CTAB, 0.005 g methylene blue and 20 gm agar (HIMEDIA) to 1 mineral salt medium (38) containing 1% glucose. The plates were incubated at 30^0C for 3 days. In another set the isolate was grown in 100 ml. MSM medium supplemented with 1% glucose, incubated at 30^0C for 3 days. Bacterial cells were removed by centrifugation (9,000 rpm for 15 min at 4°C), then added 6 N HCl (43) to the culture supernatant to obtain a final pH of 2.0, kept it at 4^0C for overnight. The acid precipitate was recovered by centrifugation (9,000 rpm for 15 min at 4°C) and was extracted with minimal amount of methanol and then filtered for removal of residues. Solvent was evaporated in a rotary evaporator under a vacuum. The dried material was dissolved in minimum volume of chloroform-methanol (1:1, vol/vol) and spotted on paper (Whatman No.1) using a chloroform-methanol-water mixture (65:25:4, vol/vol/vol) as the mobile phase. Ninhydrin solution (0.5% in acetone) was sprayed over the plate.

16S rDNA. DNA extraction and then16S rDNA genes were amplified by polymerase chain reaction (PCR) (24) using bacteria-specific 27F, 357F and the universal 1492R primers as 5´-AGAGTTTGATCCTGGCTCAG-3´, 5'CTCCTACGGGAGGCAGCAG-5' and 5´-CGGCTACCTTGTTACGACTT-3´, respectively. A gene amplifier was used to incubate reactions through an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 55°C for 30 s and 72°C for 1 min and finally at 72°C for 10 min. The PCR products with expected size of about 1.1 and 1.5 kb were pooled and purified using PCR purification columns according to the manufacturer's instructions. The 16S rDNA were sequenced by 3730 automatic sequencing equipment (ABI, US). Phylogenetic affiliation of the 16S rDNA sequence derived from the sequencing was estimated by using the program nBLAST-a tool of the National Center for Biotechnology Information.

3. Results and Discussion

Multiple PAH degrading bacteria A few colony were produced from the culture of anthracene and

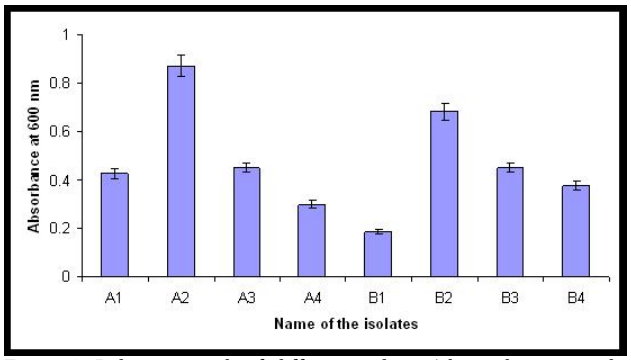

Figure 1. Relative growth of different isolates (phenanthrene as sole source of carbon)

benzo(a)pyrene rich MSM medium but none from the placebo. A total of 8 isolates, 4 from each plate were selected randomly based on their colony morphology. 6 out of 8 isolates showed better growth (degradation) on phenanthrene containing MSM medium (Fig. 1), out of which only one was growing on fluoranthene containing agar plate better. So this isolate was found to degrade anthracene, phenanthrene and fluoranthene. All experiments were repeated thrice and mean value was taken.

Bacterial growth curve A slow degradation of relatively complex hydrocarbon fluoranthene was observed (Fig. 2)

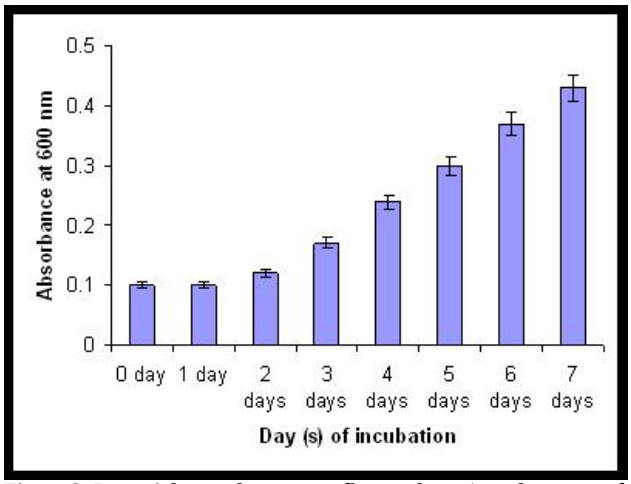

Figure 2. Bacterial growth curve on fluoranthene (as sole source of carbon)

Biochemical test The isolate was gram positive, endospore forming, rod shaped and slightly motile bacteria (Table 1). It cannot utilize lactose (Table 2) carbohydrate. Unlike many other *Bacillus* species it is highly oxidase positive (Table-3). It showed positive response to gelatin hydrolysis (Fig. 3).

Table 1: Gram stain, spore stain, IMViC test and antibiotic sensitivity test

Gram Stain	Positive
Spore stain	Endospore
Motility	Slightly motile
Ampicilin	Sensitive
Streptomycin	Resistant
Chloramphenicol	Sensitive
Fluconazole	Resistant
Indole	-
Methyl red	+
Citrate	-
McConkey agar	-

‘ +’ positive and ‘-’ negative

Gelatin zymogram The zymogram showed the presence of high molecular weight bacterial gelatinase (Fig. 4).

Table –2. Carbohydrate utilization test

Glucose	+
Galactose	+
Sucrose	+
Starch	+
Lactose	-

‘ +’ positive and ‘-’ negative

Table-3. Enzymatic tests

Nitrate reduction	+
Triple sugar iron	+
Starch hydrolysis	+
Casein hydrolysis	+
Catalase	+
Oxidase	+
Gelatinase	+
Urease	+
Lipase	+
Lecithinase	+

‘ +’ positive and ‘-’ negative

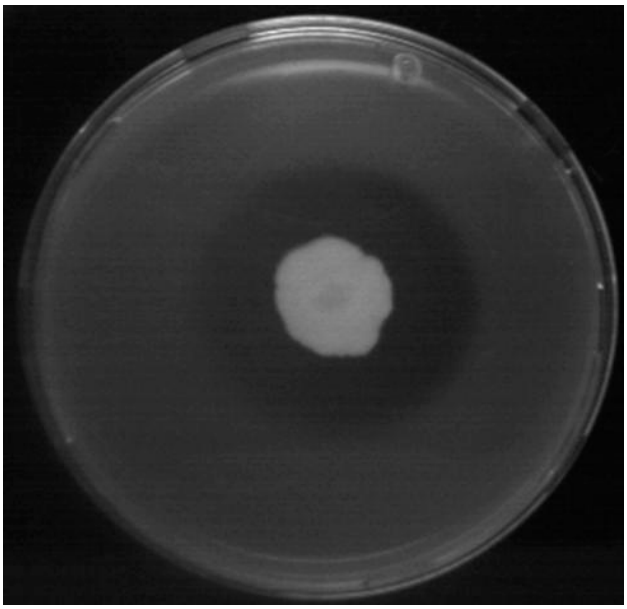

Figure 3. Gelatinase activity of the isolate

Lipase and lecithinase activity A white opaque zone surrounding the colony proved the positive test for lecithinase activity (Fig. 5). When the plate was held at an angle to the incident sunlight an iridescent (oil on water) was observed on the surface of the colony that proved the isolate was also lipase positive. A positive test for lipase activity also supports the potential of the isolate to degrade the hydrocarbons in soil (27).

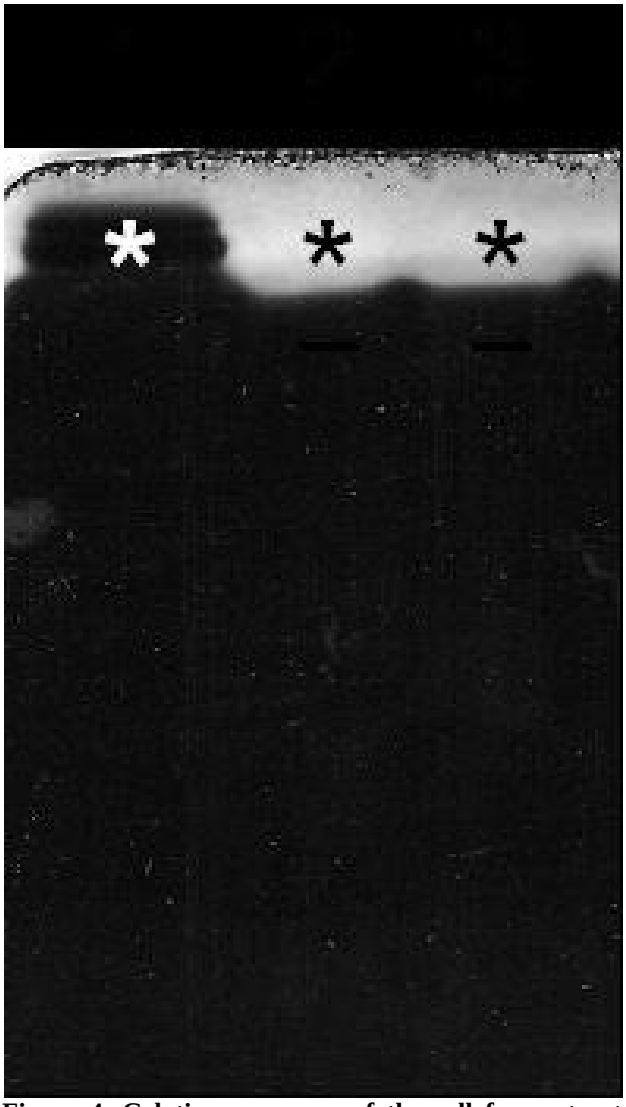

Figure 4. Gelatin zymogram of the cell free extract (Lane 1, only medium ; Lane 2 and 3 both the cell free extract)

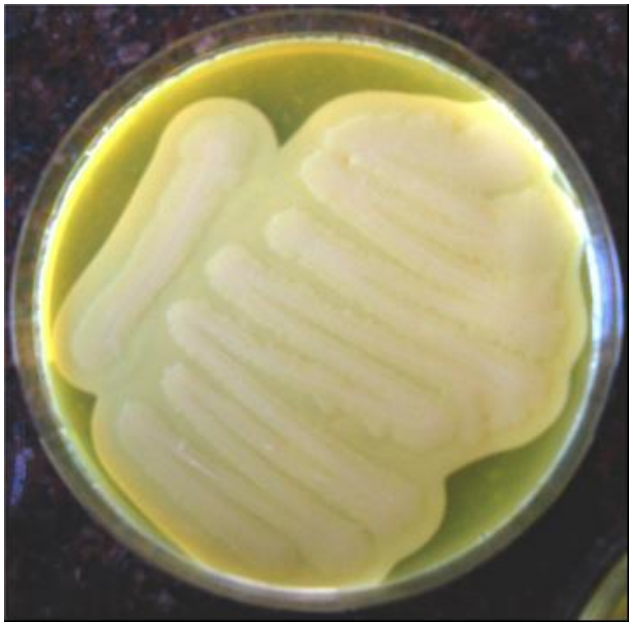

Figure 5. Lipase and lecithinase activity

Hemolytic activity RBCs surrounding the colony are degraded proving the beta hemolysis (Fig. 6). Many soil bacteria produce biosurfactant (8) that increases the bioavailability of PAHs.

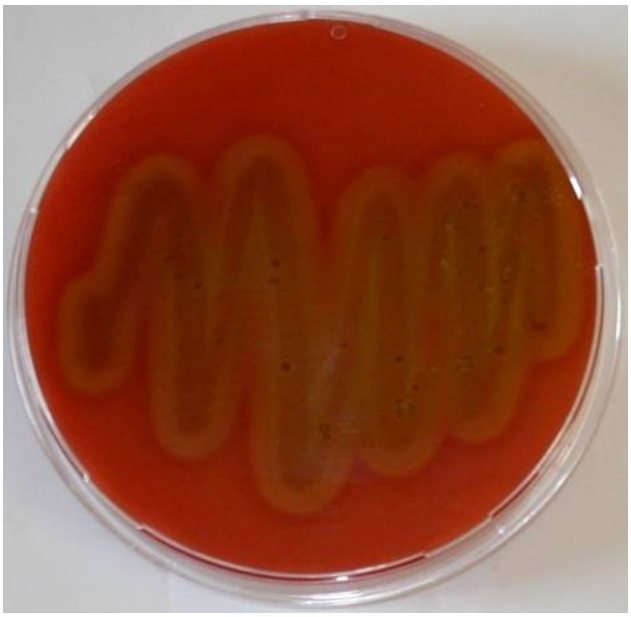

Figure 6. Hemolytic activity of the isolate resulting lysis of RBC

Measurement of surface tension The surface tension of the cell free extract decreases with time (Fig. 7).

Emulsification activity Emulsification was observed with the cell free extract (Fig. 8) but not with MSM medium alone.

CTAB-methylene blue and ninhydrin test No blue halo was formed around the colony in CTAB-methylene blue agar plate, so the biosurfactant was not anionic nature (like glycolipid type eg, rhamnolipid). But ninhydrin spray gives purple spot, so the biosurfactant may be a peptide.

Phylogenetic analysis Blast analysis with one sequence (using forward primer) showed 100% sequence

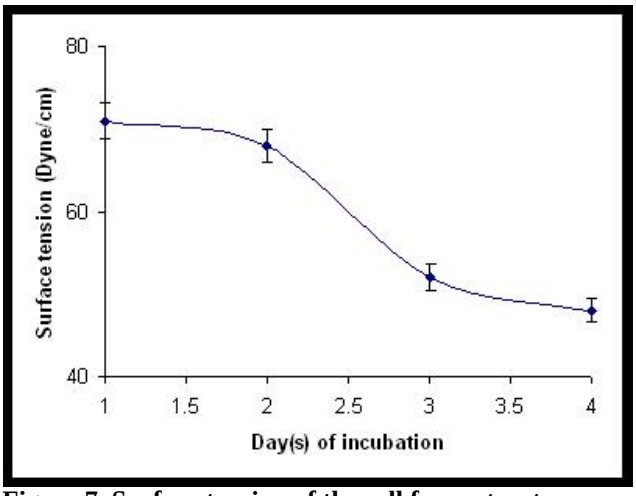

Figure 7. Surface tension of the cell free extract

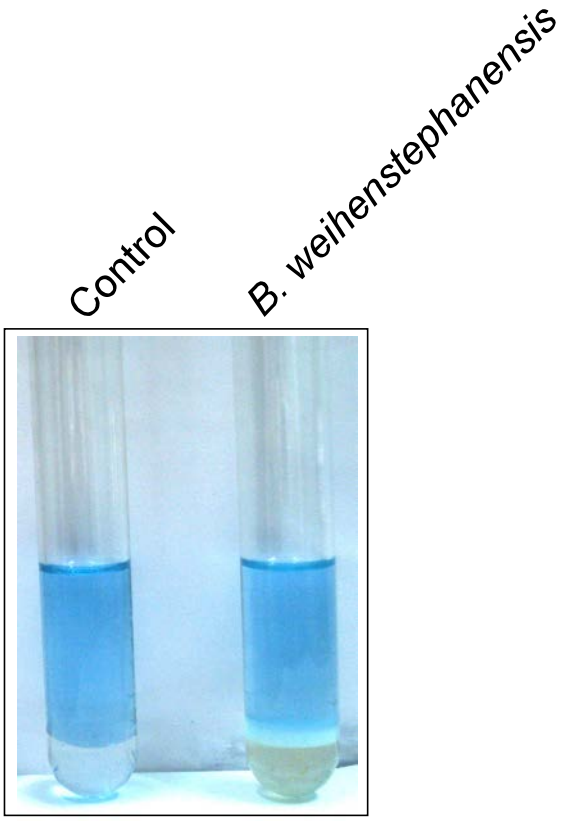

Figure 8. **Emulsification activity of the cell free extract**

homology with *Bacillus anthracis* strain ATCC 14578, *Bacillus weihenstephanensis* strain DSM11821, *Bacillus mycoides* strain 273 and *Bacillus thuringiensis* strain IAM 12077 and with another sequence (using reverse primer) showed maximum 91% sequence homology with *Bacillus thuringiensis* strain IAM 12077, *Bacillus weihenstephanensis* strain DSM11821 and *Bacillus mycoides* strain 273. Since *B. anthracis* is nonhemolytic and the strain did not grow like rhizoidal and also *B. thuringiensis* is non motile or urease negative or do not utilize sucrose, biochemical results and phylogenetic analysis prove the isolate is *Bacillus weihenstephanensis* strain DSM11821.

4. Conclusion

There are several technologies to curb the PAH pollution of the environment but that are expensive, and not ecofriendly (26). Hence, bioremediation is preferred to other methods. In this study the bacteria degrade different PAHs (anthracene, phenanthrene, and fluoranthrene). Also good biosurfactant activity and lipase activity find its industrial application (16, 35) other than bioremediation (2). High gelatinase activity of the bacteria may find its use in pest control (4). Different factors like temperature (9), pH (21), salt conc. (7, 25, 31), solvent (13), surfactant (41) can be changed to treat PAH contamination more effectively.

Acknowledgements

The work was funded by Department of Science & Technology, Government of West Bengal, India (No. 646/1(8) (Sanc.)/ST/P/S&T/Misc-14/2009 dated 29-01-2010).

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