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EXTRACELLULAR BIOCATALYTIC ACTIVITY OF THERMOPHILIC LIPASE AND PROTEASE OF *Solibacillus silvestris* NND-3

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ABSTRACT

In this present study, thermophilic Solibacillus silvestris NND-3 was isolated from hot-water spring of Atri region of Odisha state, present in India and evaluated for extracellular biocatalytic activity of thermophilic lipase and protease by using culture dependent approaches. Protease activity was observed optimum at the stationary phase, 36-hours (remain stable for 24-36 hours at 56^0C) of NND-3 isolate. Effect of organic solvents and various inhibitors and commercial detergents on residual protease activities (mean %) of NND-3 isolate were observed maximum as compared to residual lipase activity (mean %). 16S rRNA sequence of the thermophilic bacterial isolate was deposited in NCBI database and obtained GenBank accession number as JX680812. This thermophilic bacterial strain was emerged as one among few strains having high protease productivity in Firmicutes class with novel bioprospecting potential which can be used in detergent based industries.

Key Words: Thermostable lipase and protease production, 16S rRNA, thermophilic bacterial strain, extracellular biocatalytic activity.

INTRODUCTION-

Wide diversity of thermophiles having bioprospecting potential of their enzymes with numerous applications in biotechnology industries and simultaneously thermophilic micro-organisms become a novel source for study of microbiota-evolutionary relationship with other living kingdoms[1-5]. Thermophiles and hyperthermophiles belong to Firmicutes yield different hydrolytic enzymes such as celluloses, proteases, lipases and amylases which possess high industrial values[6-8].

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Culture based study of microbial diversity is tedious and time-oriented whereas modern microbiological methods for in situ study are user-friendly and less time period oriented [9]. In recent decades, microbial ecology studies give impetus to various molecular phylogenetic tools like PCR-DGGE in revealing specific ecotypes[10-12]. Bacilli class in Firmicutes contains seven diverse taxonomic lineages which are still evolving by addition of novel species[13-15]. Firmicutes consists of mostly gram-positive bacteria taxa which are not separated into coherent phylogenetic group based on phenotypic characteristics, i.e., physiology and morphology[16, 17]. Mostly beer, cider, wine spoilages are caused by Firmicutes[18].

Researchers interested in trace of novel enzymes from extremophiles due to huge demand in industrial scale by exploitation of recombinant DNA technology tools through culture dependent and independent processes of microbial prospectors[19]. Significantly, two microbial models are used to a great extent as thermophilic host, i.e., *Bacillus stearothermophilus* and *Thermus thermophilus* for production of novel industrial enzymes[20, 21]. The present work concentrates on isolation of thermophilic bacteria from Atri hot-water spring of Odisha (20^0 09`N latitude and $85^0$ 18`E longitude) and evaluate the bioprospecting potential of lipase and protease enzymes by using Winkler`s modified method (1990) and Manachini et. al. modified method (1988)[22, 23].

MATERIALS AND METHODS-

Screening of thermophilic bacterial isolate-

Water sample from Atri Hot-water spring during rainy season was collected in a sterile bottle (500ml) with pH and temperature of water at the site was measured to be 8.9 and 56ºC. Pure culture of thermophilic bacterial isolate was screened on modified Thermus Agar ATCC 697 medium using culture dependent approach. Various routine morphological characterizations and IMViC tests and catalase test were performed of thermophilic bacterial isolate (NND-3)[24].

Genomic DNA extraction, PCR amplification and 16S rDNA sequencing-

Genomic DNA of NND-3 thermophilic isolate was extracted by using XcelGen DNA isolation kit (XG2411-01) and it`s quality was evaluated on 1.2% Agarose gel. Fragment of 16S

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rDNA was amplified by PCR from above isolated genomic DNA using universal 16S rDNA primers (8F: 5` AGAGTTTGATCCTGGCTCAG-3` and 1492R: 5`-GGTTACCTTGTTACGACTT-3`) by avoiding excess freeze-thaw cycles and addition of aliquot reagents in smaller amounts[25]. On Agarose electrophoresis, single discrete PCR amplicon band of 1500 bp (Figure-6) was observed and then eluted PCR amplicon band was purified to remove contaminants by using XcelGen PCR purification kit.

Forward and reverse DNA sequencing was performed by using of Big Dye Terminator v3.0 cycle sequencing kit on ABI 3730xl Genetic analyzer. Consensus 16S rDNA sequence of 1374bp was checked by BLAST analysis and based on maximum identity score and first ten sequences were aligned with ClustalW. By utilization of MEGA 4 phylogenetic tool, the percentages of bootstrap replicate trees (partitions $\leq$50% were collapsed) within which the associated taxa clustered simultaneously in the bootstrap ascertain check (500 replicates) were shown next to the branches[26]. 16S rDNA sequences of both isolates were deposited in NCBI database and GenBank accession numbers were obtained.

Estimation of extracellular thermophilic lipase and protease production activity-

Overnight seed culture of the thermophilic bacterial isolate (NND-3) was prepared by using Czepek`s Dox medium, contained in Erlenmeyer flasks, in triplicate, at 56^0C and pH 8.9 and 165 rpm. After 24-hours of incubation, 4 ml. of each production culture were aseptically collected at an interval of 4-hours and centrifuged at 10,000 rpm at 4^0C and supernatants were assayed for lipase production activity (U/ml).

Isolated thermophilic bacterial strain NND-3 was grown in Erlenmeyer flasks with GYP medium at 56^0C for 48-hours in an orbital shaker at 165 rpm. Four ml. of medium from flask was withdrawn inside laminar air hood at a regular interval of 4-hours and the contents were centrifuged at 10,000 rpm at 4^0C for 10 minutes and protease production activity (U/ml) was checked in the supernatant.

Effect of organic solvents and inhibitors and commercial detergents on NND-3 isolate-

Residual lipase activity (mean %) was determined by modified Winkler *et al.* method (1990). To 2915 µl of 0.5 M Tris-Cl buffer, 75µl of 20mM p-NPP solution (p-nitrophenylpalmitate

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(Sigma)) was mixed and heated at 70^0C till turbidity disappeared. After cooling, 10µl of enzyme supernatants were added each time, incubated for 10 minutes and the amount of p-nitrophenol produced was measured at 410 nm using double-beam UV/VIS spectrophotometer (ECIL, INDIA), at a regular interval of 4-hours up to 48-hours. One unit of lipase activity is defined as micrograms of p-nitrophenol released from the substrate per minute per ml of enzyme.

Residual protease activity (mean %) was determined by modified Manachini *et al.* method (1988). The reaction mixture containing 4 ml. of 2.5% (w/v) casein in 50mM of Tris-HCl buffer and one ml. of enzyme supernatant was incubated for 5-minutes on ice. The enzymatic reaction was then terminated by addition of 5 ml. of 50% (w/v) Trichloroacetic acid and filtered through Whatman No. 1 filter paper and absorbance readings of the filtrate was measured at 275 nm using double-beam UV/VIS spectrophotometer (ECIL, INDIA). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µg of tyrosine/ml/min under assay conditions. A standard curve of tyrosine was plotted for this purpose.

In case of organic solvents, extracted thermophilic lipase and protease enzyme supernatants were separately mixed with 50% v/v each of methanol, ethanol, chloroform, n-butanol, diethyl ether and acetone for 2-hour at 4^oC before addition of required substrate for incubation. Similarly, in case of various inhibitors, enzyme- substrate reactions involving extracted thermophilic lipase and protease enzyme supernatants were separately performed in the presence of EDTA (5mM), PMSF (5 mM) and SDS (1 %) in reaction mixtures. In case of commercial detergents, Enzyme assays involving extracted thermophilic lipase and protease enzyme supernatants were performed in the presence of various domestic commercial detergents (1% w/v) like Ariel, Surf Excel and Rin Shakti. The residual enzyme activity was determined in each case by use of double-beam spectrophotometer.

Effect of pH and Temperature-

Thermophilic bacterial isolate (NND-3) growth was observed at 56^0C with different pH range values (5.0 – 8.9) and different temperature ranges from (45^0C-60^0C) and all bacterial culture petriplates were checked in each hour basis up to 9-hours, on a regular basis of 4-day observation.

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RESULT AND DISCUSSION-

Screening and phylogenetic characterization-

Based on morphological and biochemical test result (Table-1 and Table-2), the thermophilic bacterial isolate was designated as NND-3. For construction of phylogenetic trees, the cut-off similarity for genus and species level ranges from 97% and 99% and homology similarity[27] of NND-3 isolate was found to be 99% maximum identity with *Solibacillus silvestris* strain HR3-23 (GenBank accession no. NR_028865.1) and *Solibacillus silvestris* strain EPUK1 (Genbank accession no. JQ313581.1).The sequence alignment maximum score was 2532 with 100% query coverage (Table-3) and the phylogenetic tree of NND-3 isolate was obtained (Figure-5). 16S rDNA sequence of NND-3 isolate was submitted to NCBI database using BankIt submission tool and GenBank accession number JX680812.1 was obtained and designated as *Solibacillus silvestris* strain NND-3.

Extracellular thermophilic lipase and protease production activity (U/ml) of NND-3 isolate-

NND-3 isolate shown maximum residual lipase activity (8.8 U/ml) at the end of stationary phase, 36-hours and remains relatively stable from 24-36 hours at 56^0C and pH 8.9, in presence of olive oil (1% v/v) (Figure-1), whereas NND-3 isolate shown relatively less of residual protease activity (8.6 U/ml) at the end of stationary phase and remains less stable from 24-36 hours (Figure-2). This indicates thermotolerant lipase activity of NND-3 isolate was relatively more stable than protease activity.

Effect of pH and Temperature-

Thermophilic bacterial isolate (NND-3) shown growth at pH ranges from 6-9 with optimum pH value 9.0. But after pH 9.0, growth was ceased and thermotolerant growth ranges from (50^0C-60^0C) with temperature optima at 60^0C. This indicates about the presence of alkali tolerant capacity at high temperature of NND-3 isolate.

Effect of organic solvents and inhibitors and commercial detergents on NND-3 isolate-

In the case of effect of organic solvents, NND-3 isolate shown maximum protease activity (mean % of 70.41±1.72) on ethanol (polar solvent) and optimum on chloroform (highly non-polar solvent, mean % 66.42±2.30); acetone (more polar solvent, mean % 62.30±1.46), whereas maximum lipase activity was shown (mean % of 62.25±1.68) on diethyl-ether (non-polar solvent) as

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presented in (Figure-2). This enzymatic phenomenon indicates organic polar solvents induce hydrophobic effect at the active site of enzymes, which contributes to thermostability and prevents unfolding. Mostly, organic solvents are used for production of drugs in pharmaceutical industries[28]. Thus, in this study, the novel thermophilic protease of NND-3 isolate can be utilized for production of synthetic drug molecules.

In case of effect of inhibitors, (Figure- 3) illustrates that NND-3 isolate residual protease activity was more sensitive to SDS (1%) (mean % 69.22±1.10) than 5mM EDTA (mean % 53.22±3.46) and 5mM PMSF (mean % 22.10±0.80) at 56^0C and pH 8.9, which indicates the optimum substrate binding efficacy with anionic surfactant[29-31] and more resistant to denaturation (about 70% at pH-8.9 and temp. 56^0C) and presence of serine residue group at the active site of NND-3 protease as very less enzymatic activity observed with 5mM PMSF (a serine protease inhibitor). This thermostability efficacy with SDS proves that NND-3 isolate protease enzyme is extensively more resistant to denaturation (about 70% at pH-8.9 and temp. 56^0C) and can be utilized as novel thermophilic protease source for detergent related industries.

For a more significant approach, domestic commercial detergents (e.g., Surf Excel, Rin Shakti and Ariel) were used for evaluation of industrial potential of thermophilic lipase and protease of NND-3 isolate at pH 8.9 and 56^0C. Residual protease activity of NND-3 isolate was maximum on Surf Excel detergent (mean % 85.36±1.15) as compared to residual lipase activity (mean % 66.42±0.54), presented in (Figure-4). Surf Excel is a domestic laundry detergent designed especially use in washing machines. The use of enzymes for laundry was introduced in the early part of the 1900s by Otto Rohm, but later on availability of thermal-robust bacterial enzymes turn laundry sector to a new technology driven platform[32-36]. This salient feature of NND-3 isolate, on commercial detergents, indicates that it can be utilized as a novel candidate for protease source in detergent industries.

CONCLUSION-

Recent developments in enzyme engineering technologies led to synthesis of new bioengineered enzymes which aims to enhance their biocatalytic activity and their utilization in

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textile, leather, pharmaceutical and cosmetic industries. Now-a-days, enzyme based cleaners are become more popular because of their high biodegradability and environment friendly and thermostability nature. Researchers across the world are now more focused on use of novel biodetergents which will reduce the use of chemical detergents. A novel thermostable alkaline protease produced by *Solibacillus silvestris* NND-3, present in Atri-Hot-water spring of Odisha was found more stable against organic solvents, inhibitors, commercial detergents and can be utilized in detergent or pharmaceutical industries. Bioprospecting of novel enzyme sources present in extreme environments are vital which will help to replace old and traditional enzyme-based industrial products into more environmental friendly with high productive capacity oriented.

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COMPETING INTERSTS-

The authors declare that they have no financial or non-financial conflict of interests.

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FIGURE WITH LEGENDS-

Figure-1: Effect of different incubation time-slots on thermophilic lipase and protease production (U/ml) of NND-3 isolate.

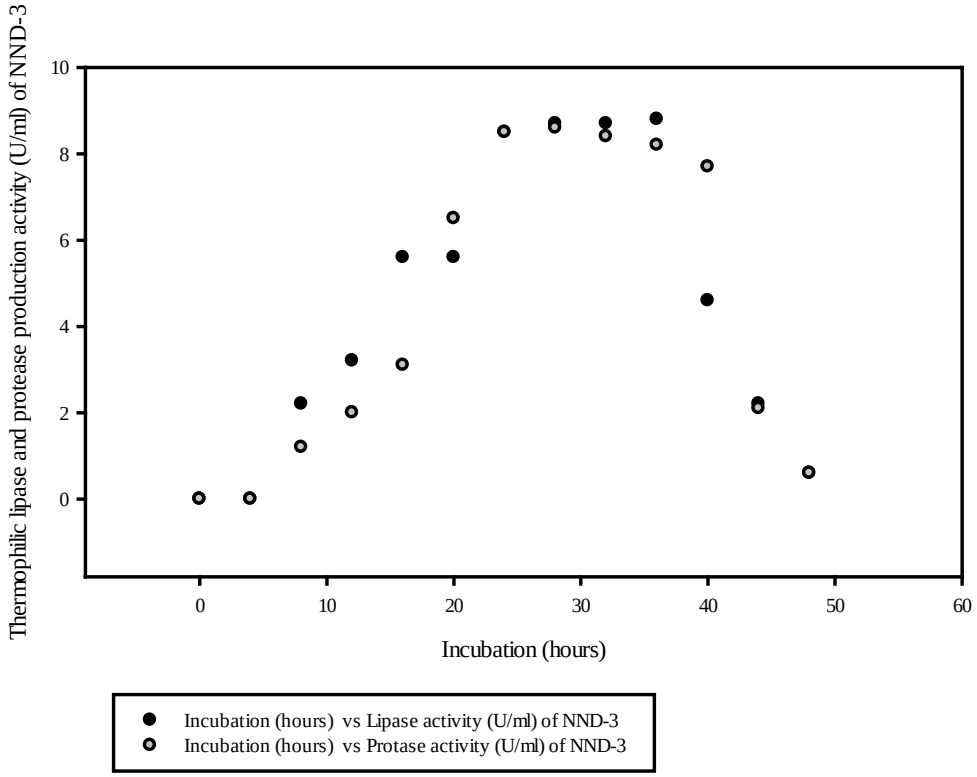

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Figure-2: Effect of organic solvents on thermophilic residual lipase and protease activity (U/ml) of NND-3 isolate.

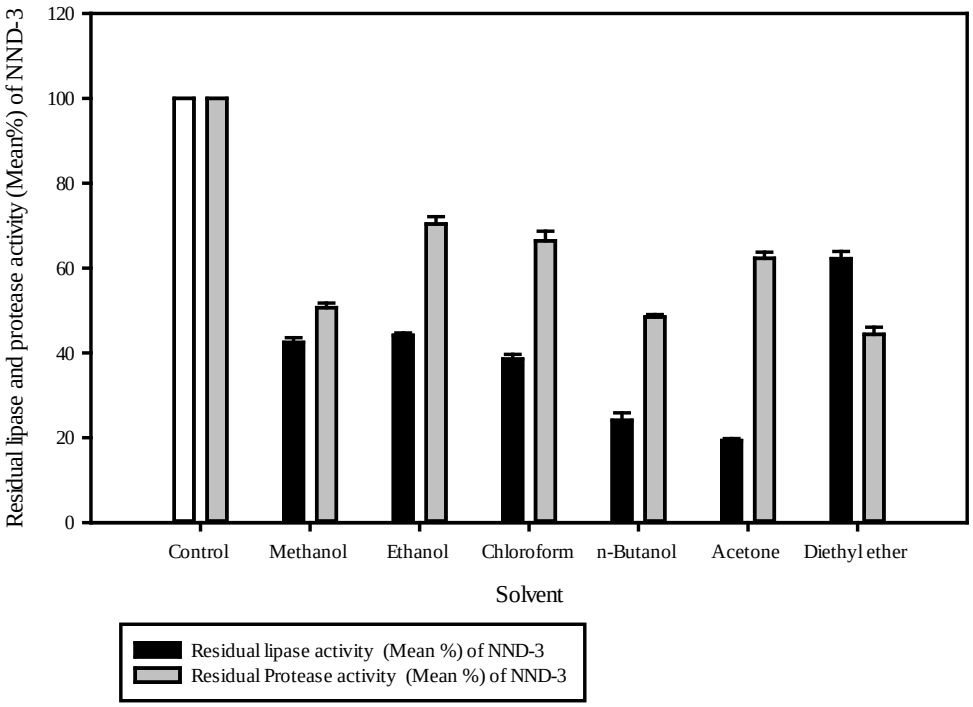

Figure-3: Effect of various inhibitors on thermophilic residual lipase and protease activity (U/ml) of NND-3 isolate.

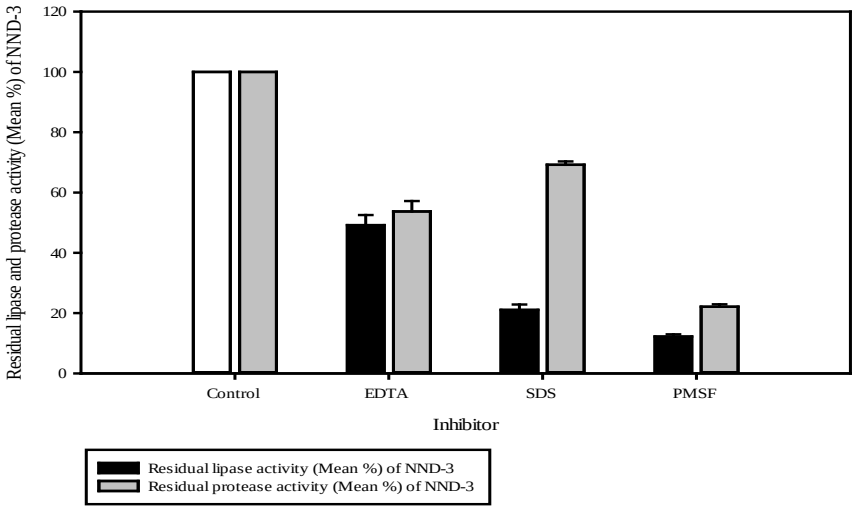

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Figure-4: Effect of domestic commercial detergents on thermophilic residual lipase and protease activity (U/ml) of NND-3 isolate.

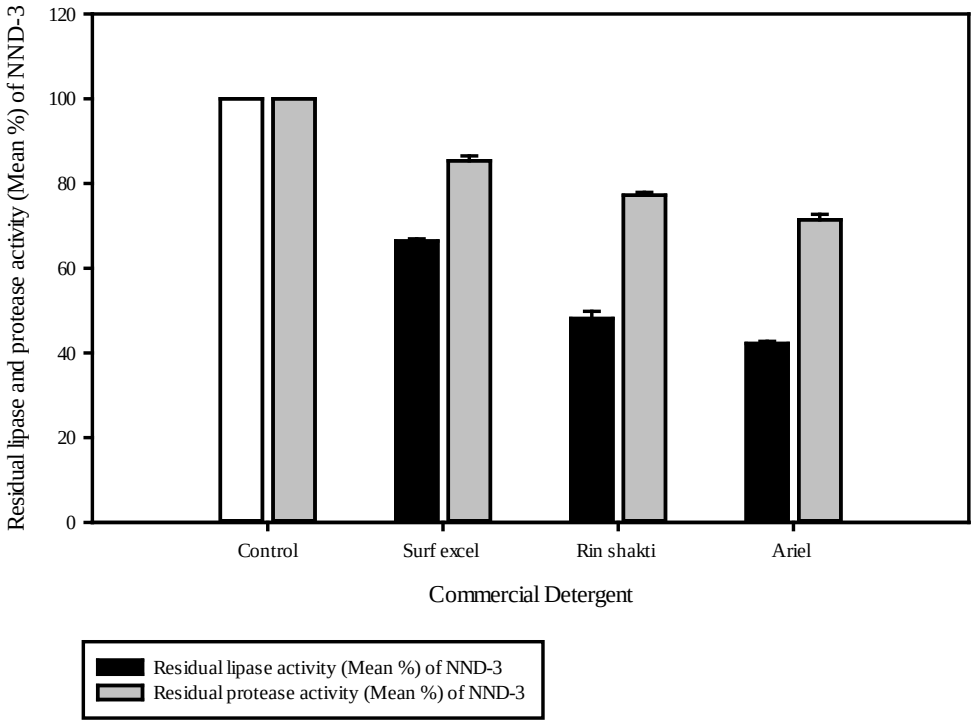

Figure-5: Phylogenetic relationships of 11-taxa containing GenBank accession No. with reference to NND-3 isolate.

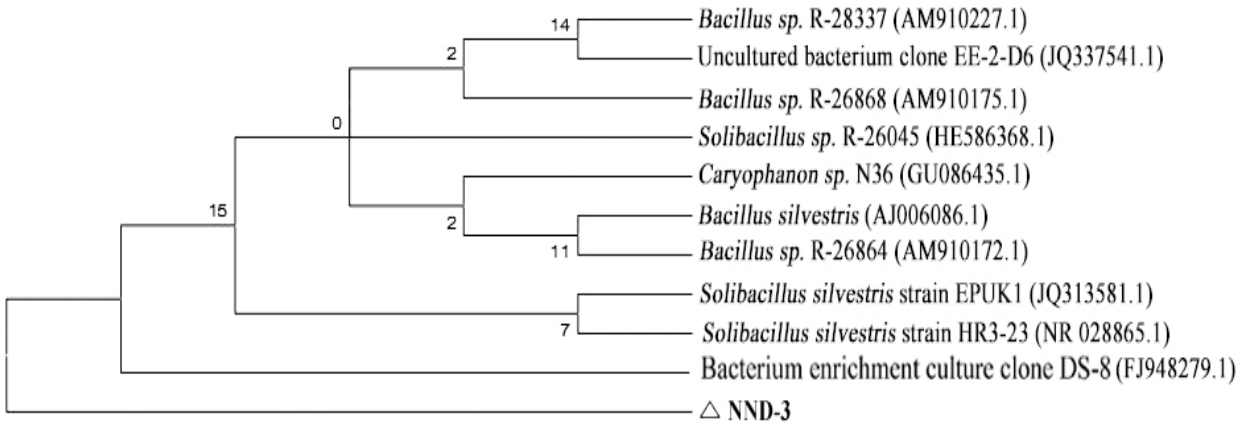

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Figure-6: PCR Amplification Gel image- (NND-3); (Lane-1: Control DNA marker); Fragment of 16S rDNA gene was amplified by PCR from isolated genomic DNA of NND-3 thermophilic bacterial isolate (using 1.2% Agarose gel electrophoresis). A single discrete PCR amplicon band of 1500 bp (Lane-2: Single DNA band of 1500 bp) was observed when resolved on Agarose Gel in each case, which was further processed for forward and reverse DNA sequencing using universal primers after removal of contaminants.

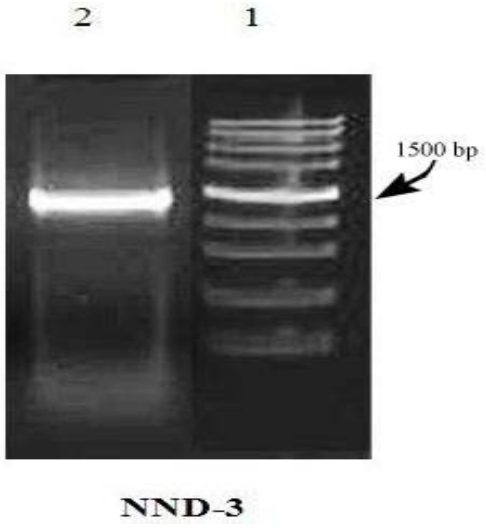

TABLE WITH LEGENDS-

Table- 1: - IMViC test and other Biochemical Test Result of NND-3 isolate

Biochemical Tests	Result (NND-3)
pH	8.9
Temperature	56^0C
Citrate Utilization	-
Lysine Utilization	-
Ornithine Utilization	-
Urease	-
Phenyl Deamination	-
Nitrate Reduction	+
H_2S Production	-
Glucose	+

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Adonitol	+
Lactose	-
Arabinose	+
Sorbitol	-
Catalase Test	+
MR-VP Test	-
Indole Production Test	-

- (+) – Positive result, (-) – Negative result.

Table-2: - Morphometric characterization of thermophilic bacterial isolates

Salient Features	NND-3
Shape	Long rods
Form	Irregular
Elevation	Convex
Colony	White
Margin	Lobate
Gram`s staining	+ ve
Motility Test	+ ve

- (+ve) – Positive, (- ve) – Negative.

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Table- 3: - Significant sequence alignments of NND-3 isolate

Accession	Description	Max Score	Total Score	Query coverage	E value	Max ident.
JQ313581.1	Solibacillus silvestris strain EPUK1	2532	2532	100%	0.0	99%
GU086435.1	Caryophanon sp. N36	2532	2532	100%	0.0	99%
NR_028865.1	Solibacillus silvestris strain HR3-23	2532	2532	100%	0.0	99%
AM910227.1	Bacillus sp. R-28337	2532	2532	100%	0.0	98%
AM910175.1	Bacillus sp. R-26868	2532	2532	100%	0.0	98%
AJ006086.1	Bacillus silvestris	2532	2532	100%	0.0	99%
AM910172.1	Bacillus sp. R-26864	2529	2529	100%	0.0	98%
JQ337541.1	Uncultured bacterium clone EE-2-D6	2527	2527	100%	0.0	97%
FJ948279.1	Bacterium enrichment culture clone DS-8	2527	2527	100%	0.0	97%
HE586368.1	Solibacillus sp. R-26045	2525	2525	100%	0.0	99%