Comparative characterization of urease secreting bacterial and fungal isolates from soil sample of farm fields

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ABSTRACT

The study was focused on to isolate the urease producing bacteria and fungus from soil sample of farm field. Bacterial isolate designated as BS-13 and fungal isolate designated as FS-13 were selected on the basis of qualitative and quantitative screening analysis for urease activity. The bacterial isolate BS-13 was Gram's positive and motile. Colony morphology of BS-13 was creamish white, flat and circular in form with undulated margins. This bacterial isolate BS-13 was positive for VP test, citrate utilization, casein hydrolysis, starch hydrolysis, oxidase, catalase but negative for indole and methyl red test. On the basis of biochemical tests and 16S rDNA sequence analysis, the BS-13 was identified as *Bacillus* species. The fungal isolate consist of dense layer of brown-black conidial heads on white-yellow mycelia base. On the basis of colony morphology, LCB staining and ITS region sequencing, the fungus was identified as *Aspergillus niger*. A 1488 bps 16S rDNA and 898 bps ITS nucleotide sequences of BS-13 & FS-02 isolates were submitted to NCBI under the accession number KM668223 and KM461718, respectively. The temperature, pH and substrate concentration, and time required for optimum production of urease by *Bacillus* sp. was 35 °C, pH 8.0, 3 mM, and 48 h respectively. The optimum incubation time period, temperature, pH and substrate concentration for production of urease enzyme by *Aspergillus niger* was found to be 120 h, 30 °C, 5.0 and 50 mM respectively. Urease production by *Bacillus* sp. and *Aspergillus niger* with their above mentioned optimized conditions showed a production of 2.52 U/ml and 3.06 U/ml, respectively at all the conditions. From the phylogenetic analysis, it has been found isolated strains *Bacillus* sp. and *Aspegillus niger* could be new strains of *Bacillus* and *Aspergillus* species.

Key words: Urease, microbes, 16S rDNA, ITS region, phylogeny.

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INTRODUCTION

Urease (EC 3.5.1.5), belongs to the super family of amidohydrolases and phosphotriesterases [1]. The best-studied urease is that from jack bean [2]. Urease from jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized [3]. Urease is a nickel-dependent metalloenzyme synthesized by plants, some bacteria and fungi. Urease enzyme is responsible for the hydrolysis

of urea fertilizers applied to the soil into NH_3 and CO_2 with the concomitant rise in soil pH [4, 5] Due to this role, urease enzyme from soil microflora have received a lot of attention, since it was first reported by Rotini [6], a process considered essential in the regulation of N supply to plants after urea fertilization. Urease enzyme in plant are predominantly intracellular [7] and microorganisms found as both intra- and extra-cellular enzymes [8, 9]. On the other hand, urease extracted from plants or microorganisms is rapidly degraded in soil by proteolytic enzymes [10, 11]. This suggests that a significant fraction of ureolytic activity in the soil is carried out by extracellular urease, which is stabilized by immobilization on organic and mineral soil colloids.

The presence of urease has been detected in numerous organisms, including plants, bacteria, algae, fungi and invertebrates. The plant and fungal ureases are mostly homohexamers α_6, whereas, bacterial ureases typically are heterotrimers $(\alpha\beta\gamma)_3$. Bacterial ureases are composed of three distinct subunits, one large (α 60-76 kDa) and two small (β 8-21 kDa, γ 6-14 kDa) subunits commonly forming $(\alpha\beta\gamma)_3$ trimers. Fungal and plant ureases are made up of identical subunits (~90 kDa each) and most commonly assembled as trimers and hexamers [12].

Urease producing soil microorganisms have been studied from a variety of bacteria and fungi. Urease producing soil microorganisms were identified as *Rhizopus Oryzae* [13], *Bacillus thuringiensis* [14], *Fusarium culmorum*, *Penicillium spinulosum*, *Citrobacter freundii*, *Enterobacter ludwigii*, *Pseudomonas chlororaphis* [15], *Actinomycete* species [16], *Aspergillus niger* [17]. Since extracellular urease plays a key role in the hydrolysis of urea, it is important to study about urease enzyme. Hence, this study was designed to identify bacterial and fungal strains from soil of farm field (Shoolini University, Solan, H.P.) in which urea based fertilizers were used regularly, which produce extracellular urease activity. Therefore the current study undertaken to isolate the bacterial and fungal isolates from farm soil for the production of extracellular urease. Biochemical characterization and their identification would shed light on type of microorganism secreting urease.

MATERIALS AND METHODS

Isolation and screening of urease producing fungal and bacterial isolates from soil sample

Soil sample was aseptically collected from the farm field of Shoolini University Bajhol, Himachal Pradesh, India. For the isolation of bacteria, serial dilution and spreading method was used. 1 g of soil was added to 9 ml of sterile distilled water and shaken vigorously. The suspension was centrifuged at 2000 rpm for 5 min to remove the insoluble particles. The supernatant was serially diluted upto 10^{-6} dilution. 100 µl of undiluted sample and 10^{-6} dilution was plated on nutrient broth (NB) containing 2 % agar and 0.2 % urea (spread plate method) for bacterial isolation, whereas it was potato dextrose agar (PDA) medium for fungal isolation.

Plates were incubated for 3 days at 37 °C for bacterial growth and at 30 °C for fungal growth. After 3 days, bacterial and fungal colonies appeared on NB agar and PDA medium respectively. Isolated bacterial colonies were purified by three successive streaking on NB agar medium and isolated fungal colonies which appeared on PDA were purified by hyphal tipping technique. Bacterial and fungal isolates were further verified by streaking on Christensen's urea agar medium at 37 °C for 72 h and growing in Christensen's urea broth at 30 °C for 4 days respectively. Quantitative analysis for extracellular urease activity was performed for bacterial and fungal isolates. For this, enzyme assay was performed for the production of ammonia from

urea by using Nessler's reagent [18]. One unit of urease activity was defined as the amount of urease that produces 1 µmole of ammonia in one min at 37 °C (0.05 M Tris-acetate buffer containing 0.2 M urea). The isolates that showed high level of urease activity were selected for further characterized.

Identification of bacterial and fungal isolates by morphological and biochemical characterization

Urease producing bacterial isolate was subjected to Gram's staining [19] and morphological and biochemical characterization according to Bergey's Manual of Systematic Bacteriology [20]. Morphological features (morphology of cell and colony) and biochemical tests (indole, methyl red reaction, Voges-Proskauer (V.P.) reaction, citrate utilization, oxidase, catalase, casein hydrolysis, starch hydrolysis and motility test) were performed for urease producing bacterial isolate. The fungal isolate was verified by Lactophenol cotton blue (LCB) staining [21].

Identification of urease producing bacterial and fungal isolates by molecular approach

For molecular identification, bacterial and fungal isolates were subjected to 16S rDNA and ITS region respectively by PCR amplification and sequencing. Genomic DNA of bacterial isolate was extracted by the method described by Sambrook et al [22]. The 16S rDNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1542R (5′-AAAGGAGGTGATCCA-3′) [23]. Genomic DNA of fungal isolate was extracted as described by Choi et al [24] and subjected to ITS amplification using the universal primers, ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) [25]. For both bacterial and fungal isolates, the amplification was done by initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min. The purified PCR amplicons of bacterial and fungal isolates were sequenced using 27F & 1542R primers and ITS 5 & ITS 4 primers, respectively. The nucleotide sequences obtained were overlaid to remove the common region and the complete nucleotide sequence of 16S rDNA was subjected to nBLAST analysis [26] against the bacterial 16S rDNA database. Similarly, the nucleotide sequence obtained for ITS was subjected to nBLAST analysis against the fungal database. The complete nucleotide sequences were submitted to GenBank database. The BLAST search matching >90% homology were selected to construct phylogenic tree. Molecular Evolutionary Genetics Analysis 6 (MEGA6) [27] was used to construct phylogenic tree using the neighbour-joining method [28] to study the molecular evolution of bacterial and fungal isolates.

RESULTS & DISCUSSION

Biochemical characterization of bacterial and fungal isolates

Microbes are known to produce urease enzyme. Urease plays an important role in maintaining the nitrogen supply to plants. It is interesting to find out whether bacteria or fungi are the main contributors of urease in the soil. Therefore, urease producing soil microorganisms have received a lot of attention. Hence, bacterial and fungal isolates from soil samples were screenedfor urease activity. .

Total 13 urease producing bacterial isolates (BS-01 to BS-13) were qualitatively screened forurease activity on Christensen's urea agar medium [Fig 1a]. Two best urease producing fungal isolates (FS-01and FS-02) were further tested for urease activity in NBmedium containing urea [Fig 2b]. Urease activity was assessed by observing the time taken to change in color of the medium from yellowish to deep pink Bacterial isolate BS-13 and fungal isolate FS-02 changed the color of the urea medium into deep pink color.

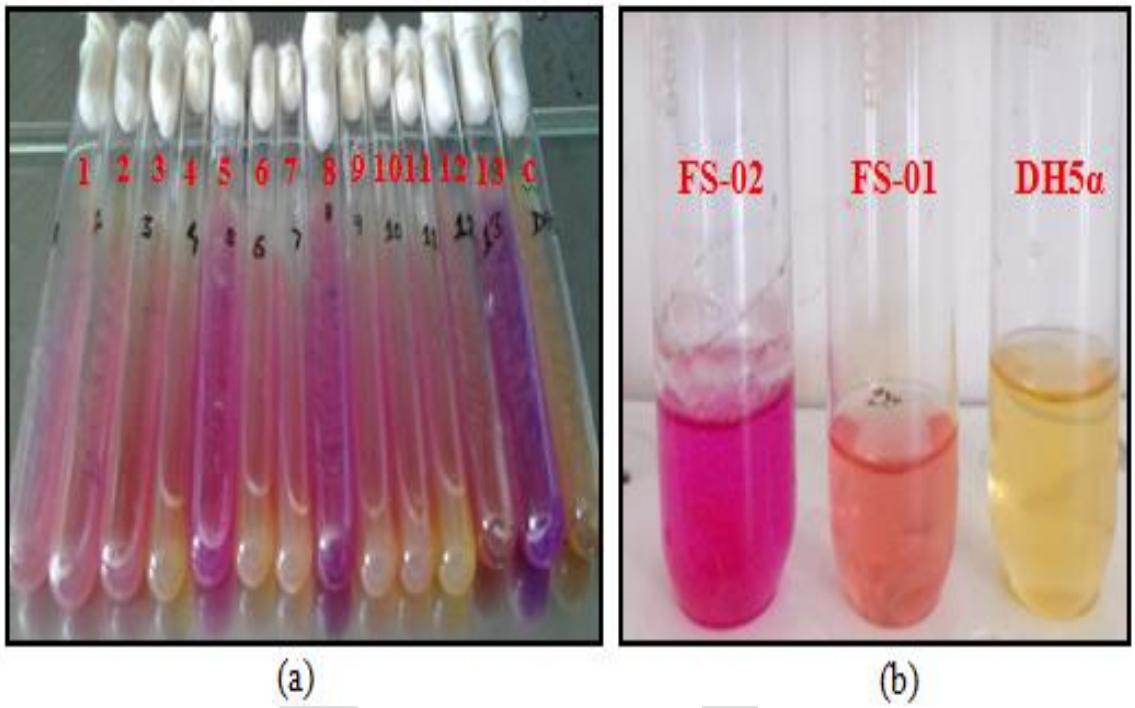

Fig 1: Screening of bacterial and fungal isolates for urease activity. Screening was done on Christensen's urea agar medium for bacterial isolates. Bacterial isolates (BS-01 to BS-13) were streaked on Christensen's urea agar medium slants. Inoculated slants were incubated at 37 °C for 4 days to visualize urease activity (a). Urea broth medium was used for screening of fungal isolates. Test tubes containing urea broth medium were inoculated with fungus strains FS-01 and FS-02 and incubated at 30 °C for 4 days to observe color change in broth medium (b)

Total 13 urease producing bacterial isolates (BS-01 to BS-13) were quantitatively screened for their extracellular urease activity by performing enzyme assay to select high urease producing bacterial isolate. Similarly, total of two urease producing fungal isolates (FS-01and FS-02) were also screened for their extracellular urease activity. Urease activity of all the isolates (bacterial and fungal) is shown in Table 1. Except BS-13, all other bacterial isolates showed very low level of the urease activity and BS-13 bacterial isolate shows more urease activity (0.88 U/ml) as compared to other bacterial isolates. In case of fungal isolates, FS-02 shows more urease activity (0.56 U/ml) as compared to FS-01 (0.105 U/ml).

Table 1. Comparison of bacterial and fungal isolates for extracellular urease activity

Bacterial and fungal Isolate		Urease activity (U/ml)
Bacterial Isolates	BS-01	0.192
	BS-02	0.125
	BS-03	0.099
	BS-04	ND
	BS-05	0.319
	BS-06	ND
	BS-07	0.137
	BS-08	0.471
	BS-09	0.072
	BS-10	0.110
	BS-11	0.161
	BS-12	0.091
	BS-13	0.880
Fungal Isolates	FS-01	0.105
	FS-02	0.561

(ND) = not detected

Hence, on the basis of both qualitative and quantitative analysis, BS-13 strain and FS-02 isolates were selected for production of urease enzyme and for other subsequent experiments.

BS-13 strain appeared singly or in chains as straight rods, Gram's positive, creamish white with undulated margins in circular form [Fig 2]. While screening, BS-13 isolate showed optimum production of urease enzyme in 24 h (0.88 U/ml) at 37 °C and pH 7.2, in the presence of 3 mM urea. Further optimization of urease enzyme was done at different time of incubation, different temperature, and pH and substrate concentration. Optimum incubation time period and temperature was found to be 48 h and 35 °C respectively for urease production by the BS-13 bacterial isolate. The optimum pH 8 and in the presence of 3 mM urea, BS-13 isolate showed urease activity of 2.52 U/ml.

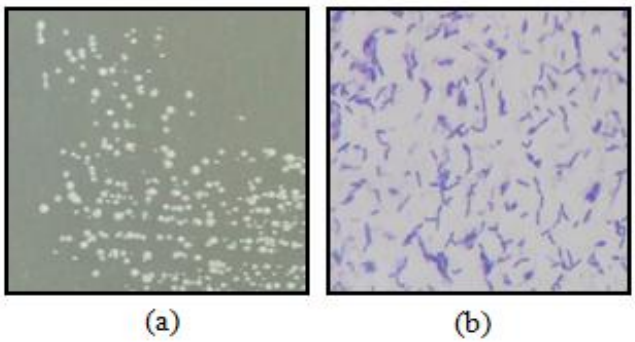

Fig 2: Morphological tests for bacterial isolate (BS-13). Colony morphology of BS-13 on nutrient agar medium (a) and microscopic examination of Gram's staining at 40X (b)

On PDA medium, colonies of FS-02 consist of a compact white or yellow basal covered by a dense layer of dark-brown to black conidial heads [Fig 3]. Conidial heads were large, dark brown to black, becoming radiate and tending to split into several loose columns with age. While screening, FS-02 showed urease production of 0.56 U/ml by 49 h in production medium at 30 °C, pH 4.3, and in the presence of 30 mM urea. To optimize the urease production by FS-02 isolate, incubation time, temperature, pH and substrate concentration of production medium was optimized. Urease activity was maximum at 120 h of incubation. Urease activity of FS-02 was maximum (3.06 U/ml) at 30 °C, pH 5, and in the presence of 50 mM urea. Seshabala and Mukkanti [29] studied the isolation of urease rich bacteria from soil sample enriched with urine (about 5 successive days) and garden soils. They reported the absence of urease rich bacteria in the garden soil. Senthil et al [30] has reported the similar observations. The strain *Klebseilla* sp. was optimized for extracellular urease production. They optimized incubation time period, temperature, pH and urea concentration for the production of urease. Urease activity (2.25 U/ml) was maximum at 35°C, pH 7.0 and 0.3% urea at 48 h of incubation.

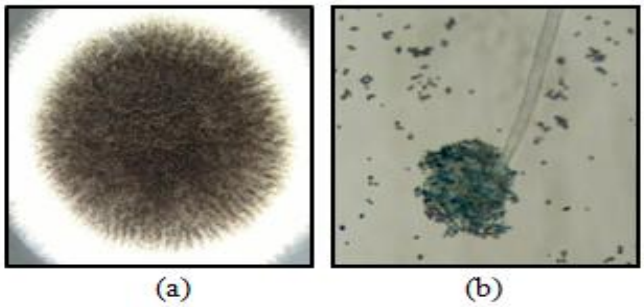

Fig 3: Morphological tests for fungal isolate (FS-02). Colony morphology of FS-02 isolate on PDA medium (a) and microscopic examination of LCB staining for FS-02 isolate at 40 X (b).

Various biochemical tests were performed to identify the bacterial isolate BS-13. It was observed that BS-13 isolate was negative for indole and methyl red test and positive for VP test, citrate utilization, casein hydrolysis, starch hydrolysis, oxidase test, catalase test and motile [Table 2] [Fig 4]. On the basis of morphological and biochemical characteristics as per the criteria of Bergey's Manual of Systematic Bacteriology [20], the bacterial isolate BS-13 was tentatively identified as *Bacillus* sp. The fungal isolate FS-02 was identified as *Aspergillus* sp. on the basis of colony morphology and microscopic examination [31]. Similarly, Das et al [32] has performed morphological identification of fungal isolate on the basis of conidia shape and arrangement of spores on the mycelia by LCB staining. They reported that the fungal isolate resembled *Aspergillus flavus* on the basis of colony morphology and microscopic examination. Senthil et al [30] studied extracellular urease production by marine bacteria. They identified *Klebsiella* spp, *Proteus* spp, *Lactobacillus* spp and *Streptococcus* sp. by biochemical methods with the help of Bergey's Manual of Determinative Bacteriology. Chatterjee et al [14] has reported similar observations. They reported two urease producing isolates of *Bacillus thuringiensis* (BTc 152 and BTc 175) isolated from soil of rice field of Tarakeswar of India. Both urease producing isolates were white, flat and circular in form. BTc 152 was with undulate margins and BTc 175 was with entire margins. Both isolates were positive for Gram's staining, catalase, and VP tests and non-motile. Bolton et al [33] has identified 10 isolates of urease-positive thermophilic *Campylobacter* (UPTC) organisms from the natural environment. These UPTC strains were gram negative and oxidase, catalase, and nitrate positive.

Table 2. Biochemical tests for bacterial isolate (BS-13)

Biochemical Tests	Results
Indole test	Negative
Methyl red test	Negative
VP test	Positive
Citrate utilization test	Positive
Motility test	Positive
Oxidase test	Positive
Catalase test	Positive
Casien hydrolysis	Positive
Starch hydrolysis	Positive

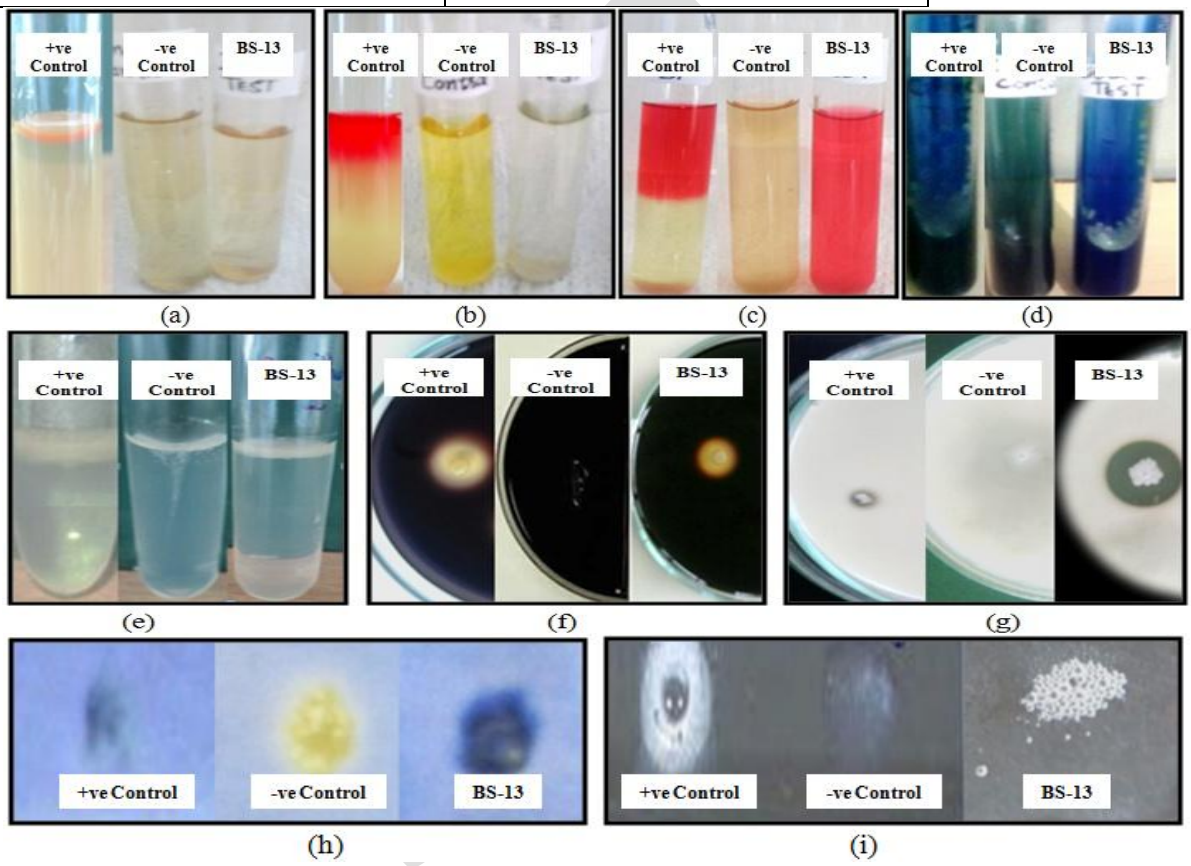

Fig 4: Biochemical tests for bacterial isolate (BS-13). (a) Indole test, *E. coli* strain DH5α was used as positive control and *Pseudomonas fluorescens* was used as negative control. (b) Methyl red test, *E. coli* strain DH5α was used as positive control and *Enterobacter aerogenes* was used as negative control. (c) VP test, *Enterobacter aerogenes* was used as positive control and *E. coli* strain DH5α was used as negative control. (d) Citrate utilization test, *Enterobacter aerogenes* was used as positive control and *E. coli* strain DH5α was used as negative control. (e) Motility test, *E. coli* strain DH5α was used as positive control and *staphylococcus aureus* was used as negative control. (f) Starch hydrolysis test, *Bacillus subtilis* was used as positive control and *E. coli* strain DH5α was used as negative control. (g) Casein hydrolysis test, *Bacillus subtilis* was

used as positive control and *E. coli* strain DH5α was used as negative control. (h) Oxidase test, MTCC 8115 was used as positive control and MTCC 8515 was used as negative control. (i) catalase test, MTCC 8515 was used as positive control and MTCC 8114 was used as negative control

Molecular identification of bacterial and fungal isolates

Genomic DNA of BS-13 isolate and FS-02 isolate was subjected to PCR amplification and separated on 1% agarose gel. A PCR product of ~1500 bps was detected for BS-13 isolate, whereas it was ~900 bps for FS-02 isolate as indicated [Fig 5 a and b]. The results of sequencing revealed a nucleotide sequence of 1488 nucleotides for BS-13 isolate and 898 nucleotides for FS-02 isolate. 16S rDNA sequence of BS-13 and ITS sequence of FS-02 was subjected to nBLAST analysis against 16S rDNA bacterial and fungal ITS database respectively. On the basis of 16S rDNA studies, the BS-13 isolate showed 98% similarities with *Bacillus* sp. (Accession no. KJ588869). From these results, the BS-13 strain was identified as *Bacillus* sp., and submitted under GenBank Accession number KM668223. Similarly, on the basis of morphological characteristics and ITS studies, the FS-02 strain was found to be *Aspergillus* sp., and showed 98% similarity with *Aspergillus awamori* (Accession no. KJ660725). From these results, the FS-02 strain was identified as *Aspergillus niger* and submitted under accession number KM461718. Varenyam et al [34] isolated two urease producing bacterial strains (CT2 and CT5) from highly alkaline cement samples using the enrichment culture technique. On the basis of various physiological tests and 16S rRNA sequence analysis, these bacterial strains were identified as *Bacillus* species. Akhtar et al [35] isolated five fungal urease positive strains from fruits and vegetables. On the basis of molecular analysis, they reported that these urease producing isolates were *A. niger* and genetically all these five strains were more than 99.5% similar with each other as determined by their nucleotide alignment using NCBI tools.

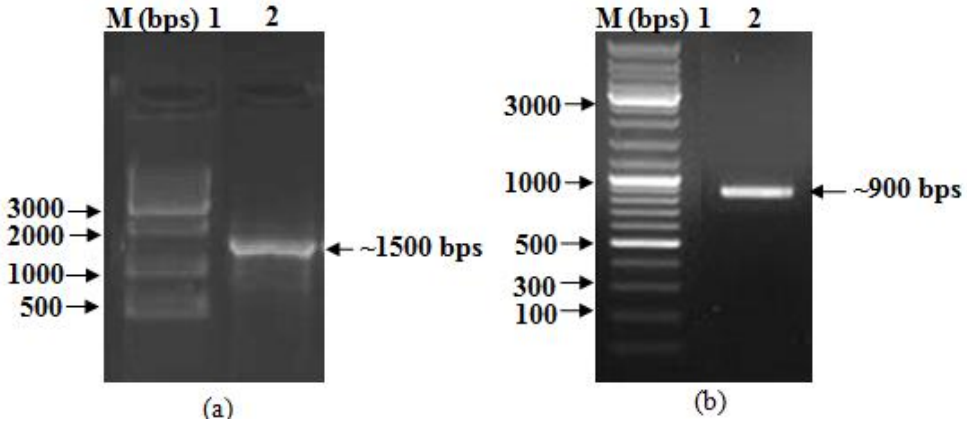

Fig 5: Amplification of gene encoding 16S rDNA of BS-13 (a) and ITS region of FS-02 (b)
PCR amplified products were separated on 1% agarose gel and visualized under Gel document system (Alpha Innotech). Lane 1 is a molecular size marker (bps), whereas Lane 2 is a PCR amplified product of BS-13 and FS-02 in panel a and b respectively.

Phylogenetic analysis of bacterial and fungal isolates

For both bacterial and fungal isolates, phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis 6 (MEGA6) [27] using the Neighbour-Joining Method [28].

From phylogenetic analysis, it has been found that there is no genepool sharing of bacterial strain *Bacillus* sp., KM668223 with other strains of *Bacillus* species that were used to construct phylogenetic tree. *Bacillus* sp. (KM668223) representing more genetic distance from other strains of *Bacillus* species [Fig 6]. Thus, isolated strain could be unique and a new strain of *Bacillus* species. Similarly, there is no genepool sharing of *Aspergillus niger* (KM461718) isolate with other strains of *Aspergillus* species that were used to construct phylogenic tree [Fig 7]. Similarly, Das et al [32] performed the molecular identification of a mold isolate by partial 18S rDNA sequencing. 18S rDNA nucleotide sequence of 702 bps was obtained and compared with available 18S ribosomal sequences in the NCBI database using nBLAST and reported that isolate shares a same clade with *Aspergillus flavus* and occupies a distinct phylogenetic position within the representative members of the genus *Aspergillus*.

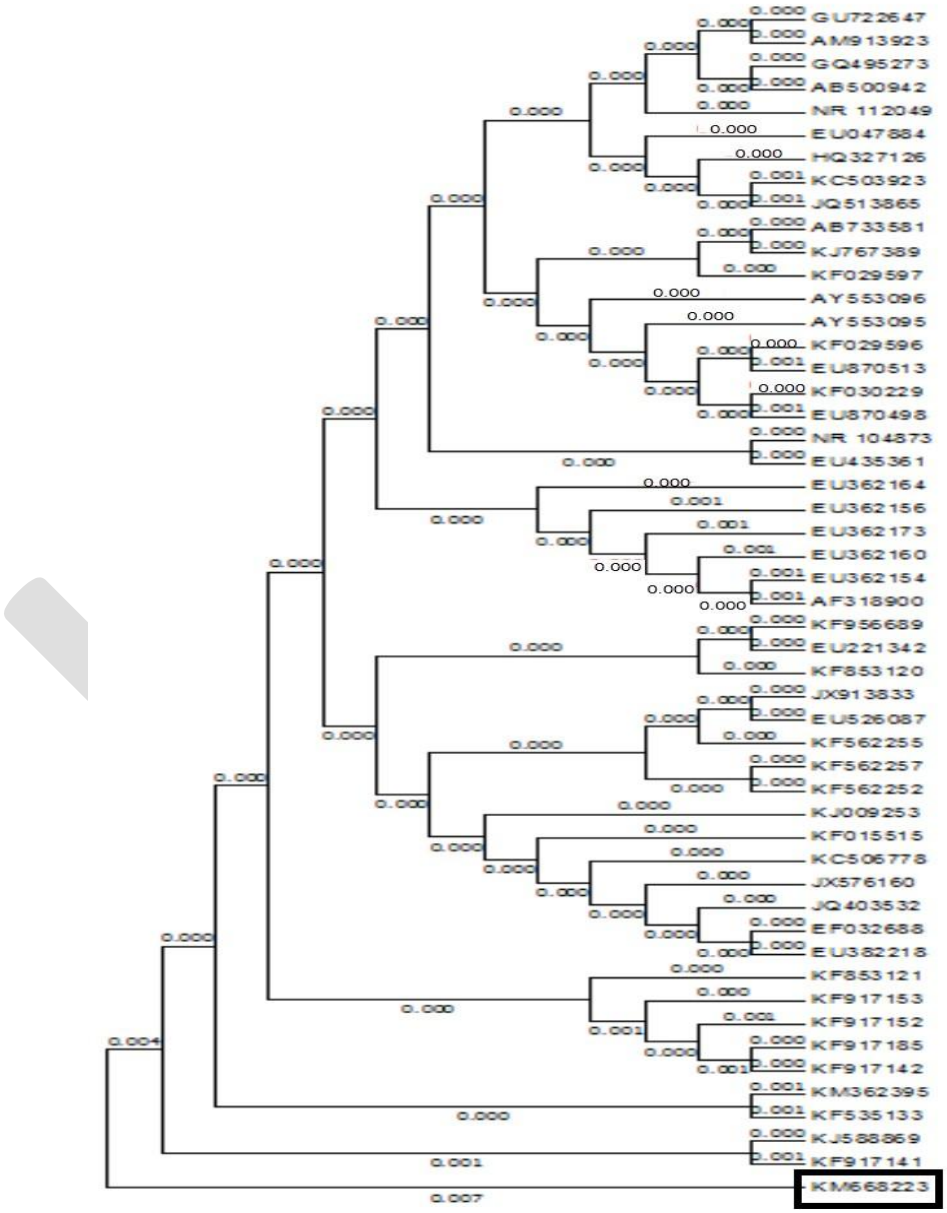

Fig 6: Phylogenic evolution of strain *Bacillus* sp., BS-13(KM668223)

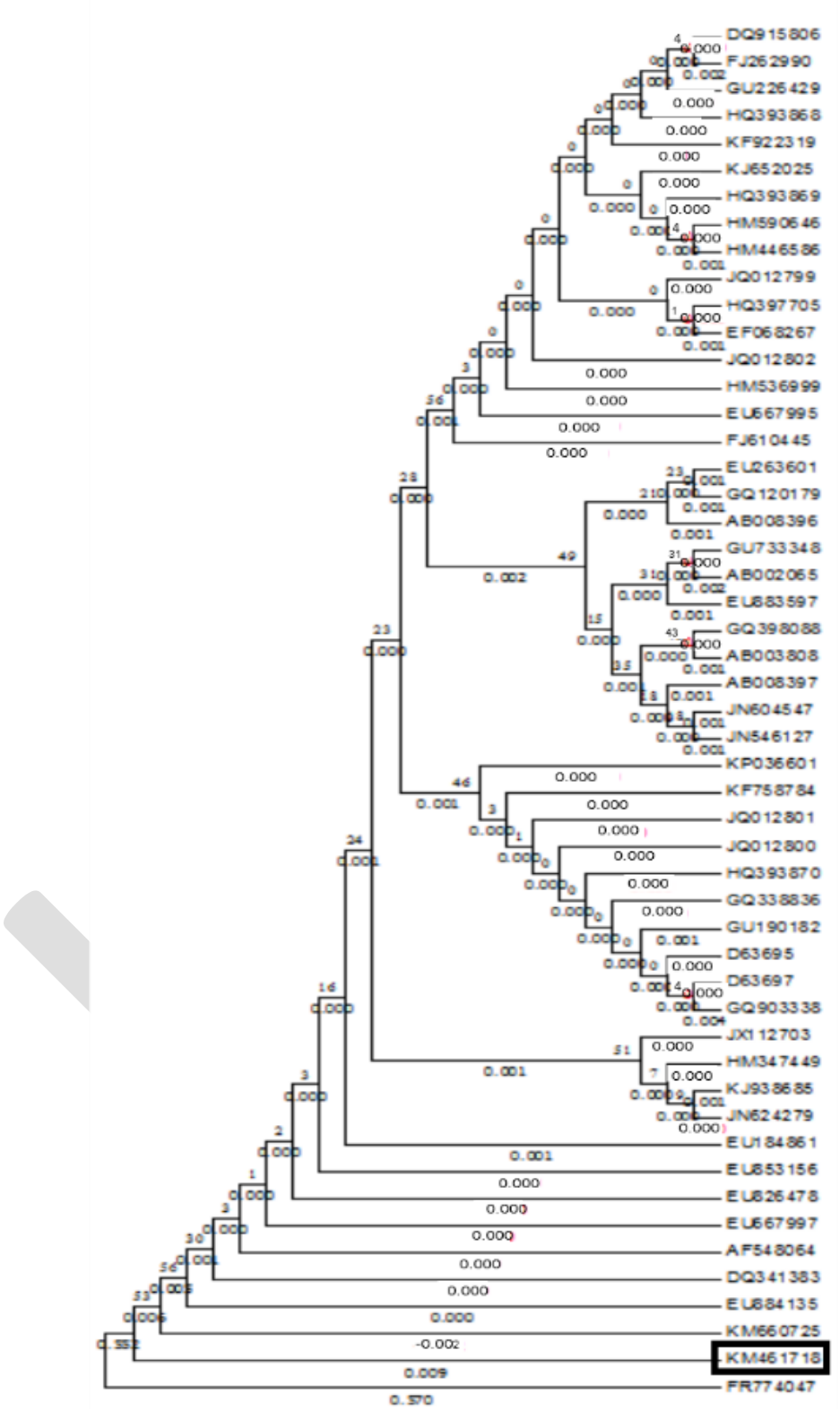

Fig 7: Phylogenic evolution of *Aspergillus niger*, FS-02 (KM461718)

CONCLUSION

The present study is the assessment of better extracellular urease producing bacterial and fungal isolates. Bacterial isolate was identified as *Bacillus* sp. and fungal isolate was identified as *Aspergillus niger* and produce urease enzyme of 2.52 U/ml and 3.06 U/ml respectively. Both BS-13 and FS-02 isolates occupied a distinct phylogenetic position within the representative members of the genus *Bacillus* and *Aspergillus*.

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