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In silico and *in vitro* Study of Two Novel Closely Related Biogas Digestate Bacilli Strains

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Abstract: Biochemical, morphological and molecular variance in *Bacillus subtilis* and *B. tequilensis* is reported here. Gen Bank-submitted partial rRNA sequences of KIIT VSKC006 (K6; KF410850.1) and KIIT VSKW003 (K3; KF410851.1) isolated from biogas digestate while formulating effective microbial consortia, upon being subjected to a BLAST search, revealed that they belonged to *Bacillus*. These rRNA sequences with high maximum-identity-score values were selected and aligned using multiple alignment software ClustalW. Phylogenetic trees constructed using MEGA 5 (neighbour-joining) revealed K6 and K3 as *B. tequilensis* and *B. subtilis* respectively. BioEdit analyses for the similarity in the gene sequences through the constructed nucleotide composition graph revealed that, the percent GC and AT contents (K6: 55.09, 44.91; K3:55.22, 44.78, respectively) and the molecular weights (single-stranded; 3897 d) in both the strains differed. Global alignment of the sequences using Needleman-Wunsch algorithm (EMBL-EBI) showed a 98.9 % identity match. When grown on agar at 30°C for 96 hrs, K6 showed irregular, cream colonies whereas K3 exhibited circular, off-white colonies with prominent concentric circles throughout. When re-grown in nutrient broth, the growth patterns were visibly different; K6 showed white, clumped, suspended particles whereas K3 displayed uniform turbidity. These *in vitro* and *in silico* analyses infer that, the difference in the two reported isolates was obvious, to the extent of being different species though closely related.

Key words: *Bacillus*, BLAST, GenBank, MEGA, Phylogeny

Introduction

Bacillus comprises a diverse number of hardy Gram-positive, motile, spore-forming rods, majority of which are non-pathogens, thus making them an ideal candidate for a wide range of applications. *B. subtilis* that produces an array of significant enzymes against a wide range of substrates commonly encountered in the environment [1], is one such well-researched species. The endospore-forming ability of bacilli aid in tolerating extreme environmental conditions; under most conditions they remain as biologically inactive spores [1,2]. The phylogeny, classification, identification and nomenclature of the two bacilli (*B. subtilis* and *B. tequilensis*) remain methodologically and logically distinct. The taxa attached together in the phylogenetic tree imply their descent from a common ancestor. Using computational phylogenetics methods, molecular phylogenetic trees are constructed with a number of input sequences. Distance-matrix methods, *viz*., neighbour-joining and UPGMA, which compute genetic distance from multiple sequence alignments, are some of the popular ones. Sequence alignment methods such as ClustalW use simpler algorithms based on distance for the construct [3,4].

Biogasification, anoxic biotransformation of biodegradable material to methane, involves

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microbially-mediated four steps, *viz*., hydrolysis, acidogenesis, acetogenesis and methanogenesis [5], at least the last three steps of which involve specialised microbes. It has wide applications, from waste management to biofuel generation. Floating-drum biogas plant, held in position by a guiding frame, comprises of a digester mounted with a moving gas-holder. The gas-holder floats directly on the fermentation slurry, or in a water jacket of its own. The accumulating gas collects in this movable gas-holder. The gas is supplied at a constant pressure, and the volume of the stored gas can be estimated by the position of the gas-holder [6]. Operated on a continuous basis, the operation is scalable, commonly employed at 5-15 m^3 (small- to middle-sized farms), 20-100 m^3 (institutions and larger agro-industrial estates), or still larger digester sizes at a community level. Advantages of such plants are that they are easy to operate. However, the two major technical bottlenecks in technology upgradation are, the multi-step bioprocessing involved, and each of the process step being driven by specialised microbial groups. Thus, attempts are on to formulate microbial consortia capable of producing an array of biocatalysts to address the issue, and thereby enhance biogasification. While assaying the microbial isolates from the kitchen refuse fed digestate, out of a whole range of them, two encountered bacilli were subjected to detailed *in silico* and *in vitro* study.

Methodologies

Both *in vitro* (wet-lab) and *in silico* (dry-lab) studies were conducted to confirm the findings based on the primary data obtained, and corroboration with the published literature.

In vitro (wet-lab) activities

To unravel potent niche-specific effective microorganism (EM) candidates from microbial consortia, two unknown isolates (*viz*., K6 and K3) with accession numbers KF410850.1 and KF410851.1 respectively, possessing diverse morphology, and capable of degrading major kitchen refuse components like cellulose, starch and lipid, were randomly picked from operational family-size floating-dome kitchen refuse fed biodigesters. To study the growth characteristics in broth, the strains were incubated in nutrient broth overnight. To decipher the morphological and the growth pattern characteristics, the cultures were repeatedly grown on nutrient agar plates at 30°C in duplicates, and observed for four days at a 24 h time lag. Further, the isolates were subjected to selected biochemical tests to compare their metabolic uniqueness. To compare the isolates at molecular level, the RNA contents were isolated from both the strains using Phenol-Chloroform extraction method.

In silico (dry-lab) activities

The partial 16S rRNA gene sequencing was obtained from third party (Xcelris Pvt Ltd, Hyderabad) bioservices provider, done by Sanger dideoxy sequencing technology. BLAST search was carried out using the partial rRNA sequence with the database of NCBI GenBank. The sequences were confirmed that they are from the plus strands by hybridising with other reported similar sequences. The nucleotide sequences of closely reported strains, *viz*., *B. subtilis*, *B. tequilensis*, *B. licheniformis*, and *B. mojavensis* in case of K6 and *B. subtilis*, *B. tequilensis*, and *B. amyloliquefacians* in case of K3 as obtained from BLAST search were aligned using multiple alignment software ClustalW. Phylogenetic tree was constructed using MEGA 5 (neighbour-joining)[7,8]. Evolutionary distances were computed using the Kimura 2 parameter method [10]. Bioedit [11] and global alignment tool using Needleman-Wunsch algorithm in Pair Wise sequence Alignment (EMBL-EBI) were further used to determine the GC % and rate of similarity between the two strains, respectively.

Results and Discussion

Characteristics

The diversity in morphology on nutrient agar plates and the growth patterns in the nutrient broth cultures observed at 24 h time lag till the fourth day revealed that K6 grew better. Though both the cultures initially grew as typical small round colonies, K6 exhibited irregular, cream colonies whereas K3 exhibited circular, off-white colonies with prominent concentric circles by 96 h (Fig.

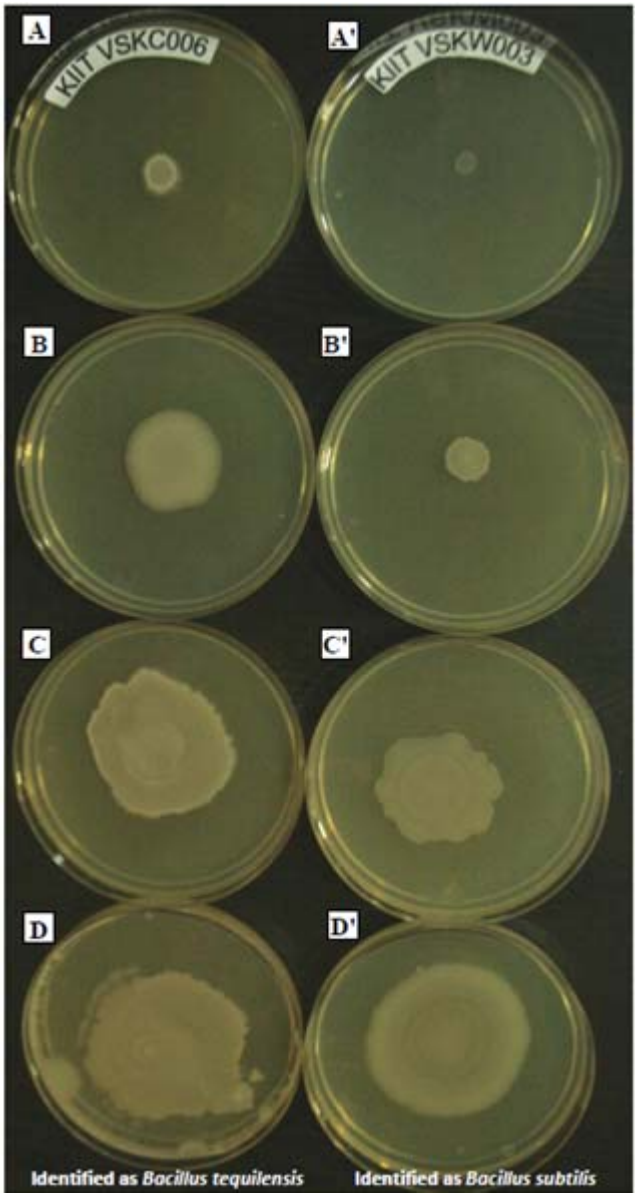

Fig. 1. Colony morphology of the isolates till 4th day (**K6**: A–D and **K3**: A′–D′) on 24-h time lag bases

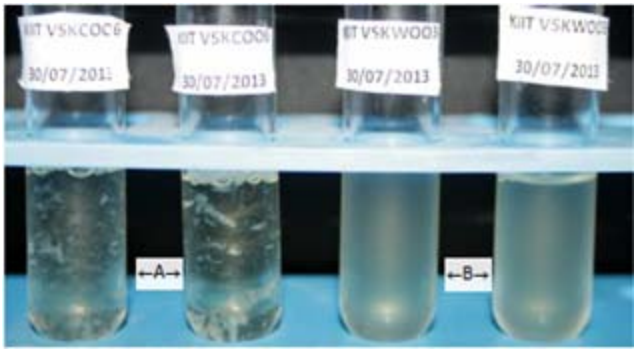

Fig. 2. Growth Pattern of K6 (**A**) and K3 (**B**) in nutrient broth after 24 h of incubation

1). Upon repeated culturing in nutrient broth, K6 showed white, clumped, suspended particulate growth whereas K3 exhibited uniform turbidity (Fig. 2) every time. This visibly different growth patterns inferred that the strains must not be one and the same. Further, the biochemical and cultural conditions comparison showed appreciable differences among the two (tentatively considering K6 as *B. tequilensis*, and K3 as *B. subtilis*). Both the strains can grow well in aerobic conditions, whereas K3 could grow optimally and K6 exhibited lesser growth under anaerobic conditions. Both the strains showed positive results for amylase, cellulase, lipase and citrate utilisation.

As one of the prominent anomalies, K6 showed positive and K3 showed negative results for tryptophan (indole) tests. The comparisons have been presented in Table 1. Further, inconsistencies were observed while reviewing some published reports. As for instance, Gatson *et al.* [2] discussed that *B. tequilensis* did not decompose starch in the reported table while mentioned the reverse in text. For a molecular level confirmation, the isolated and sequenced partial rRNAs were analysed by the means of several bioinformatics tools.

BLAST

The BLAST search identified the two isolates as closely related to *Bacillus* query coverage with *B. subtilis* showing 100 % identity. K6 was closely related to *B. tequilensis* and *B. mojaven-sis* and K3 with *B. tequilensis*. Suspecting that the E-value might have skewed the results, the BLAST was repeated by decreasing the value from the default 10.0 gradually to 0.001, showing no variation in the returned values.

Phylogenetic analyses

The phylogenetic trees constructed in MEGA 5 reported K6 and K3 as close to *B. tequilensis* strain S2Y2-a (accession number JQ828865.1), and *B. subtilis* strains XGL205, CSBR-E BACI4 (accession numbers JQ062993.1 and AB726089.1) respectively (Fig. 3). In MEGA 5, four different confidence intervals (or bootstrap values) of 500, 1000, 1500, 2000 were considered to generate phylogenetic tree which showed no significant differences in terms of evolutionary relationship [9]. It was inferred that K6 and K3 strains were related to *B. tequilensis* and *B. subtilis* respectively. Aguiar and Schrago [13] demonstrated that, under different assumptions, the simulated sequences produced biased assessment of SM (supermatrix), ST (supertree) and SppT (species tree) methods regarding topology, rates and evolutionary model [13].

Table 1. Comparison of some characteristics between *B. tequilensis* and *B. subtilis*

Characteristics	K6 (*B. tequilensis*)	K3 (*B. subtilis*)	References
Morphology			
Colony pigmentation	Cream	Off-white	17
Colony Opacity	Opaque	Opaque	17
Colony Shape	Irregular	Circular	Present report
In liquid Broth	White, clumped, suspended particles	Uniformly turbid	Present report
Growth conditions			
Aerobic	Positive	Positive	17,12
Anaerobic	Positive (Less)	Positive (Optimal)	17,12
Biochemical analyses			
Tryptophan (Indole test)	Positive	Negative	2
Starch hydrolysis	Positive	Positive	2
Cellulase	Positive	Positive	Present report
Lipase	Positive	Positive	15
Citrate utilization	Positive	Positive	2

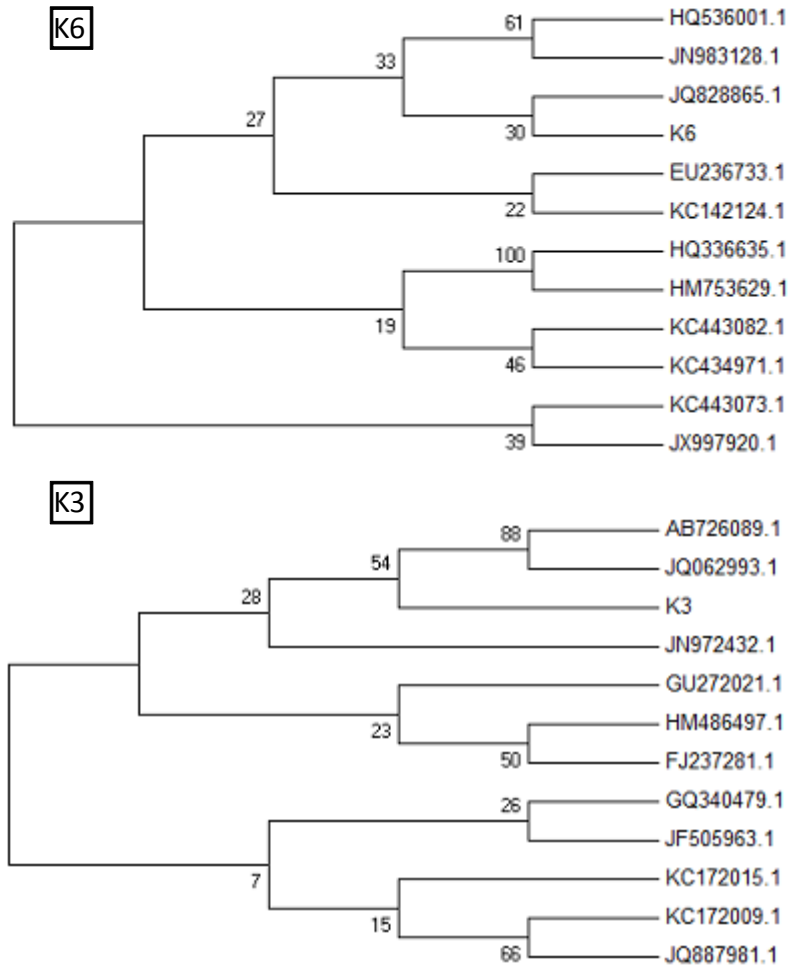

Fig. 3. Phylogenetic tree constructs using neighbour joining method in MEGA 5

Confirmatory analyses

On account of the results given by MEGA 5, the sequences were compared to ascertain their similarity through BioEdit through constructed nucleotide composition graphs (Fig. 4). It revealed that the percent GC and AT contents in either strain varied. The percent GC contents in K6 and K3 were 55.09 and 55.22, agreeing with Claus and Berkeley [14] who reported that the GC content of *Bacillus* strain must fall roughly in the range of 33-65. The respective percent AT contents thus would naturally be 44.91 and 44.78. The difference in lengths of the two sequences (rRNA) was 13 bps, and in molecular weight (single-stranded) was 3897 d. Thus, the two sequences have to be different, similar to the observations of Gatson *et al*. [2] who reported that *B. tequilensis* and *B. subtilis* had 99.3 % similarity in the 16S rRNA sequences, but not the same in any case.

Further, the global alignment of the two sequences using Needleman-Wunsch algorithm in Pair Wise sequence Alignment (EMBL-EBI) showed a 98.9 % identity match between the sequences. From the above comparison it is obvious that the two gene sequences are almost similar signifying that the strains are closely related, but not identical. Published works revealed that the partial 16S rRNA nucleotide sequences of both *B. subtilis* and *B. tequilensis* were closely similar, though not the same. The colony morphologies, biochemistry, DNA-DNA hybridisation, PFGE qualify them as different [1,2,15,16,17,18].

Conclusion

Based on the colony morphology, biochemical

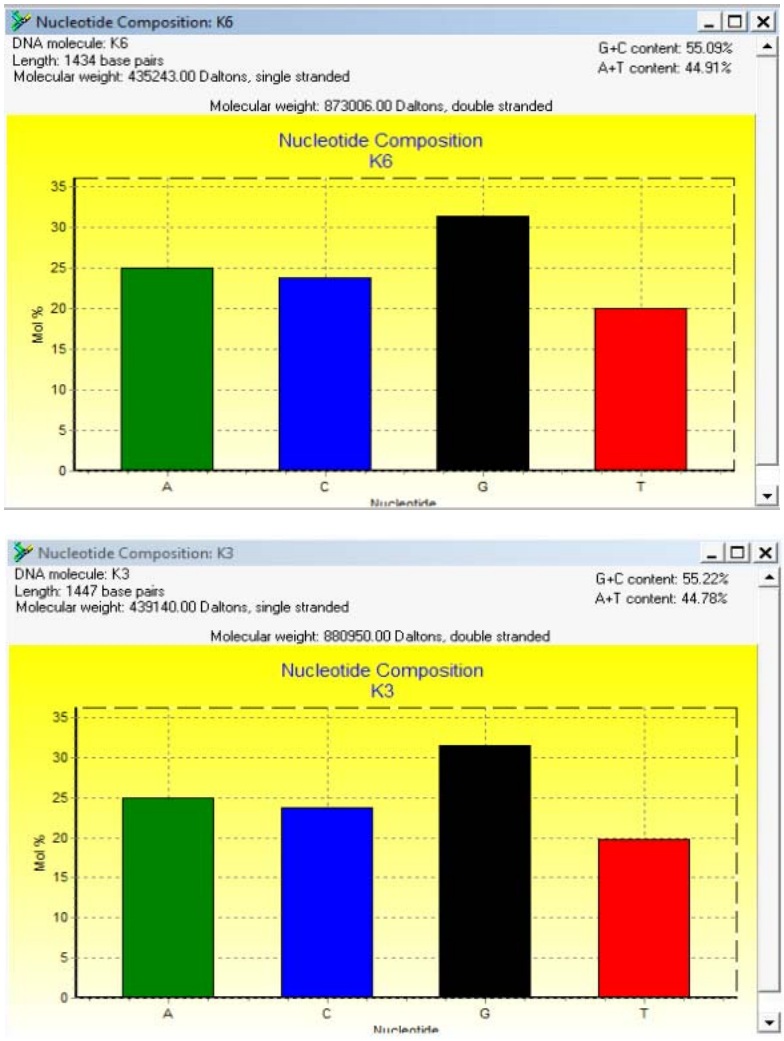

Fig. 4. Nucleotide composition in BioEdit of K6 (above), and K3 (below)

analyses, growth rates and patterns, and the systematic bioinformatics analyses, K6 and K3 were identified as *B. tequilensis* and *B. subtilis* respectively, differing from each other at the various morphological and biochemical levels but closely related at the molecular level. The partial 16S rRNA sequences of both the strains were only 98.9 % identical with 13 bp difference in length, 3897 d difference in molecular weight, and 55.09-55.22% difference in the GC contents.

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