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ORIGINAL ARTICLE

Molecular identification of a *Bacillus cereus* strain from Murrah buffalo milk showed *in vitro* bioremediation properties on selective heavy metals

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ABSTRACT

Objective: This study aims for molecular identification of naturally growing *Bacillus cereus* strain from a unique source, able to survive, and alleviate heavy metals from the nature.
Materials and Methods: Pure isolate from Murrah buffalo milk was prepared in *B. cereus* selective Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar (PEMBA) medium through a cascade of contamination free subcultures. The morphological and biochemical tests were done prior to 16S rRNA gene sequencing for strain identification and further physiological tests. The test strain was inoculated in both solid and suspension culture medium supplemented individually with Cd, Cu, Ag, and Zn to reveal the qualitative and quantitative heavy metal tolerance properties, respectively. Finally, the data collected from the *in vitro* assessment was statistically analyzed
Results: Molecular analysis revealed that the test strain was *B. cereus* BF2, which was motile, catalase positive and Gram positive rod. *B. cereus* BF2 was found significant at 0.3% bile salt tolerance [two-way analysis of variance (ANOVA)—p value is < 0.0001] where, t-test p value is < 0.0002 between Control Group (CG) and TGR-1; $p < 0.037$ between TGR-1 and 2; $p < 0.0014$ between CG and TGR-2. Similarly, *B. cereus* BF2 was significant in pH tolerant up to 8.0 with $p < 0.0115$ (in scale $p < 0.05$). The heavy metal tolerance test revealed that the test metals could not stop the growth of *B. cereus* BF2 even after 24 h of incubation but partially suppressed the growth kinetics for letting into stationary phase. Among the four heavy metals, Cd and Zn showed partial antagonism to the growth of *B. cereus* BF2. The survivability was highly significant in the medium supplemented with Zn ($p < 0.0001$) and Ag ($p < 0.018$).
Conclusion: *Bacillus cereus* BF2 can survive in selective heavy metals with metal resistance and biodegradation capacity.

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Introduction

Heavy metals are naturally occurred metal groups that can work as contaminants for ecosystem if deposited in high amount in nature. Mining, surface finishing industries, air or water pollution, milling are the principal emergence of heavy metal pollution. Of late, excessive bioaccumulation of heavy metals can render massive adversity to the living beings [1]. They are toxic, mutagenic, and carcinogenic. Micro-organisms growth, metabolism, and differentiation outright or obliquely linked with metals. A myriad of bacteria showing its tolerance at different concentrations of heavy metals [2,3]. For the capacity of bioaccumulation and resistance property assessment on differentiated metal ions, isolation, identification, and necessary characterizations are required. *Bacillus cereus* has this type of marvelous retention. *B. cereus* strains are acquainted to dwell in soil and food as motile, facultative anaerobic, spore forming, Gram-positive rods; considered severe food spoiling pathogen which often consequence non-gastrointestinal-tract infections at diversified fatality range. Some *Bacillus* spp. occupy in extreme environment, namely, *B. badius, Bacillus subtilis*, and *B. cereus* [4]. *Bacillus* spp. has already proved potentially antagonistic to pathogens, such as *Escherichia coli* and *Staphylococcus aureus* [5].

Often the range of pH, bile salt concentration, and organic–inorganic entities affect heavy metal toxicity

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on microorganisms and even influenced with the facts of speciation [6]. Some probiotic bacteria can also tolerate heavy metal toxicity at different stressed conditions. *Lactobacillus fermentum* SN_4 and *Lactobacillus rhamnosus* SN_6 have exhibited survivability against heavy metals [7]. According to Kirillova et al. [8], few *Lactobacillus* strains showed their cadmium and lead removability. In the same way, *L. fermentum* and *L. plantarum* revealed the same property of bioremediation [8].

In our experiment, whether or not different entities and concentrations of metals (cadmium, copper, silver, and zinc) play roles on the test strain of *B. cereus* survivability was scrutinized and the metal fortitude level of *B. cereus* strain of our interest was analyzed. The ultimate aims of our investigation were regulated through isolation, identification, and characterization of a new *B. cereus* strain from a unique source and the profiling of heavy metal tolerance property of the strain so that a new micro-organism can unveil its potentiality to the list of established bioremediation agents and can be a good choice for the next generation bioremediation tool.

Materials and Methods

Isolation of presumptive B. cereus

Milk sample of a Murrah buffalo (from Haryana, India) was collected from Government Buffalo Farm, Bagerhat District, Bangladesh using Nordic Iceberg. Primary culture was prepared on Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar (PEMBA) medium selective for *B. cereus* [9] at 37°C for 24 to 72 h from the 11th step of serial dilution of the milk sample. Following that, seven times consecutive contamination-free subculture were commenced to prepare pure isolate. Finally, similar to different looking 10 single colonies from the final pure culture plate were taken for further characterization separately.

Morphological and biochemical tests

The morphological tests considered the study of the bacterial size, shape, and motility status, while gram staining and catalase test were for biochemical tests of the presumptive pure isolates [10] exhibiting probiotic properties. The best result showing colony was elected for 16S rRNA gene analysis to identify the exact strain embedded inside.

Molecular identification of the test strain

16S rRNA sequencing were undertaken through RNA extraction, 1.2% Agarose Gel Electrophoresis, isolated RNA amplification with Universal 16S rRNA Specific Primer 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (AAGTCGTAACAAGGTAACC) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single amplified polymerase chain reaction (PCR) band of ~1400 bp was obtained for enzymatically purified for Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplification was carried out with 8F and 1401R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Finally, the gene sequence of the target isolate of our interest was submitted to NCBI through Gene Bank and the new accession number was registered. Analysis of the evolutionary relationship using MEGA5 was done following Neighbor-Joining method as referred by Saitou and Nei [11].

Physiological tests

Bile salt and pH tolerance test at 10 different concentrations with two replications of procedure for each test were undertaken following [12].

Heavy metal tolerance test

The test strain was cultured in PEMBA medium supplemented with $3CdSO_4.8H_2O$; Ag_2SO_4, $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$ (each with 0.05%, 0.15%, and 0.5% concentration) to observe bacterial growth patterns through naked observation. Besides, Polymyxin pyruvate egg-yolk mannitol–bromothymol blue (PEMB)-broth media containing the aforementioned metal salts at the same concentration were prepared separately and the target strain was inoculated for incubation at 37°C for 24 h. The optical density (OD) value was taken every 12 h interval by UV-Vis Spectrophotometer UV-1280 (Shimadzu) to identify the growth response of the test strain to the concentrations of heavy metals over time.

$$\mathrm{OD} = \log_{10} \frac{I_0}{I}$$

I_0 = incident optical intensity; I = transmitted optical intensity

Statistical analysis

The statistical analysis of the data collected from OD observation was performed using statistical analysis system and GraphPad Prism 8.

Results and Discussion

The effect of bile salt concentration on B. cereus BF2

Based on the morphological and biochemical characteristics (catalase test, gram staining test, oxidase test etc.), the isolate was clearly identified as *Bacillus* spices. Different fluctuations were observed when employing a series of bile concentration at different times. As a control of the study, broth with 0% concentration of bile salt was selected. Table 1 illustrates the survival rate of bacteria at ten different bile salt concentrations (0.1%–1.0%). The highest survival capability for Treatment Group Replication-1 was seen at 0.5 and 0.8 concentration of bile salt. At higher

Table 1. Survivability of *B. cereus* BF2 at different concentrations of bile salt and various pH levels.

Bile Salt (%)∞	OD_{650}			pH∞∞	OD_{650}	
***	CG[a]	TGR-1[b]	TGR-2[c]	**	R^1	R^2
0.1	0.129	0.133	0.144	1	0.797	0.799
0.2	0.133	0.14	0.156	2	0.432	0.43
0.3	0.133	0.147	0.164	3	0.333	0.334
0.4	0.132	0.147	0.166	4	0.356	0.356
0.5	0.133	0.148	0.161	5	0.359	0.361
0.6	0.134	0.143	0.154	6	0.366	0.369
0.7	0.134	0.142	0.151	7	0.38	0.381
0.8	0.133	0.148	0.139	8	0.398	0.399
0.9	0.133	0.143	0.137	9	0.169	0.17
1.0	0.135	0.135	0.132	10	0.148	0.149

∞ Grouped data analysis of the two-way ANOVA reports- *** $p < 0.0001$ (which is highly significant to the scale $p < 0.05$) for the bile salt tolerance test; a,b,c are all significant values (in scale $p < 0.05$); [a] $p < 0.0002$ between CG and Treatment Group Replication-1 (TGR-1); [b] $p < 0.037$ between the TGR-1and 2; [c] $p < 0.0014$ between CG and TGR-2
∞∞ *t*-test reports- ** $p < 0.0115$ (significant to the scale $p < 0.05$) in the pH tolerance test data considering the two replications (R)

concentration of bile salt (1.0), the lower survivability of *B. cereus* BF2 was depicted. In case of Treatment Group Replication-2, higher tolerance level was seen in the presence of 0.4 bile salt concentration. Gradual increment of bile salt concentrations resulted in gradual decrease of the bacterial growth rate as well as their tolerance level [13].

The effect of acid tolerance test

Various pH levels (1–10) were selected with *B. cereus* BF2 to check their growth and survival capacity in stressed condition. At pH 2 and 9, sudden fall in their growth was observed than the initial growth rate. At pH 10, the lower growth rate was recorded as growth decreased with the pH increment. The similar finding was noticed in the investigations of Thomassin et al. [14] regarding *B. cereus* proliferation. Browne and Dowds [15] experiment was also analogous with our finding.

In another experiment, the result recorded no bacterial growth below pH 5 and growth developed when the pH gradually increased [16]. Such a phenomenon also previously described by Everis and Betts [17] in case of *B. polymyxa* and *Clostridium tyrobutyricum*. *B. thuringiensis* was found to grow well at pH 4.0–7.0 [18]. Some experiments [19,20] deduced that the food acidity causing *B. cereus* better grew in the range of minimal p^H (4.5%–5.15%).

Molecular identification and phylogenetic analysis

Agarose gel Electrophoresis was used for analysis of PCR result. The Figure 1 exhibits the band of 16S rRNA gene of *B. cereus* on 1.2% gel electrophoresis when observed under trans-illuminator. The size of the PCR product was 1,356 bp measuring with the ladder of 2 kb.

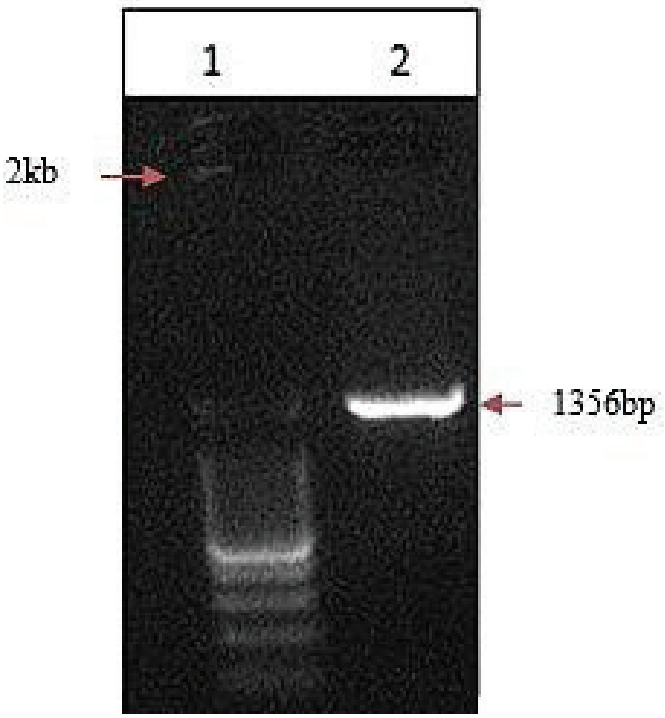

Figure 1. 1.2% Agarose gel electrophoresis showing 16S rRNA amplicon of *Bacillus cereus* BF2 at lane 2 where, lane 1 indicates 2kb ladder.

The targeted gene sequencing revealed that the strain was *B. cereus* BF2 (GenBank Accession No. MH569091.1). The phylogenetic tree has covered 12 different bacterial strains. Identification of the target strains was completed following its higher similarities to the reference strains

in the Gene Bank. The phylogenetic lineage of *B. cereus* BF2 was compared with the sequence of *B. cereus* st2, *B. anthracis* AN8, *B. cereus* LOCK 1002, *B. anthracis* LOS6, *B. thuringiensis* Ou2, *B. thuringiensis* ML 233, *B. cereus* ZLynn1000-13, *B. thuringiensis* ZLynn1000-39, *B. subtilis* B7, *Enterobacter cloacae* SZ2, and *E. cloacae* Y219 from NCBI.

Three different strains of *B. cereus* were found with their maximum similarities with *B. cereus* BF2, including *B. cereus* st2, *B. cereus* LOCK 1002, *B. cereus* ZLynn 1000-13 (Fig. 2). *B. anthracis* LOS6 and *B. thuringiensis* Ou2 were also closely related to different species. In contrast, there is distant relationship between *B. cereus* BF2 and *E. cloacae* strains. Different researchers found similar relationship among different strains of *B. cereus* and *B. thuringiensis* in their phylogenetic tree analysis [21,22].

The phylogenetic analysis through the Neighbor-Joining method as referred by Saitou and Nei [11]. Following Felsenstein [23], the bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. The branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed [23].

Analyzing the survivability against heavy metals

The evaluation of growth of *B. cereus* BF2 against different heavy metals was done in either solid and suspension culture conditions (at 0.05%, 0.15%, and 0.5%). For qualitative analysis, solid PEMBA media supplemented with heavy metals (Cd, Cu, Ag, and Zn) provided no distinguishable suppression on the bacterial growth except Cd and Zn supplementation. In contrast, the quantitative assessment by OD from the suspension culture showed different growth pattern in heavy metals survivability at 550 nm in different time interval (Fig. 3). The two-way ANOVA test revealed "*p*" values at 0.6737; 0.31; 0.018, and 0.0001 (in scale of significance $p < 0.05$) in $3CdSO_4.8H_2O$ (3a); $CuSO_4.5H_2O$ (3b); $AgSO_4$ (3c); and $ZnSO_4.7H_2O$ (3d), respectively.

The Figure 3a and d demonstrates that *B. cereus* BF2 continued its growth even after 12 h incubation when 0.05% of Cd and Zn were applied. At 24 h of incubation with 0.5% concentration of both of the heavy metals, the bacterial strain started entering slight stationary phase losing the growth kinetics. In the Figure 3b and c, *B. cereus* continued its growth after 12 h when subjected to Cu and Ag, while the growth pattern eventually increased after 24 h at various concentrations. Results of the experiment pointed out that the strain entirely survived on Cu and Ag and continued their growth on those heavy metals for longer periods. According to Behera et al. [24], Cd and Cu had more toxic effect on the *B. cereus* growth. The findings of Kalantar [25] were quite similar with us where they reported that when the concentration of Cd increases the *Bacillus* spp. growth declines. But, the result differed in some studies [26,27], where the *B. cereus* grew well in Cd comprising media.

In case of Zn, some studies on *Bacillus* spp. demonstrated their findings [28,29] that high concentration of Zn showed depletion on bacterial growth. The result of the experiment of Khande et al. [30] showed similarity to our findings. According to Ghahfarokhi et al. [31], Gram-negative bacteria showed significant survivability with Ag at different concentration which supports our outcomes.

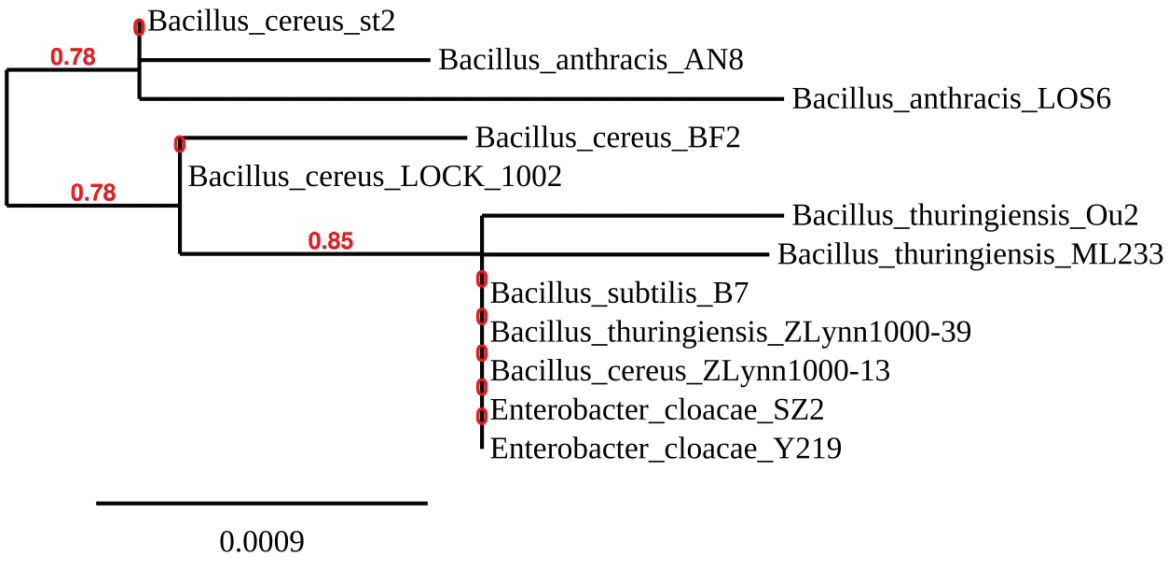

Figure 2. Evolutionary relationship of *Bacillus cereus* strain BF2 (Accession No. MH569091.1).

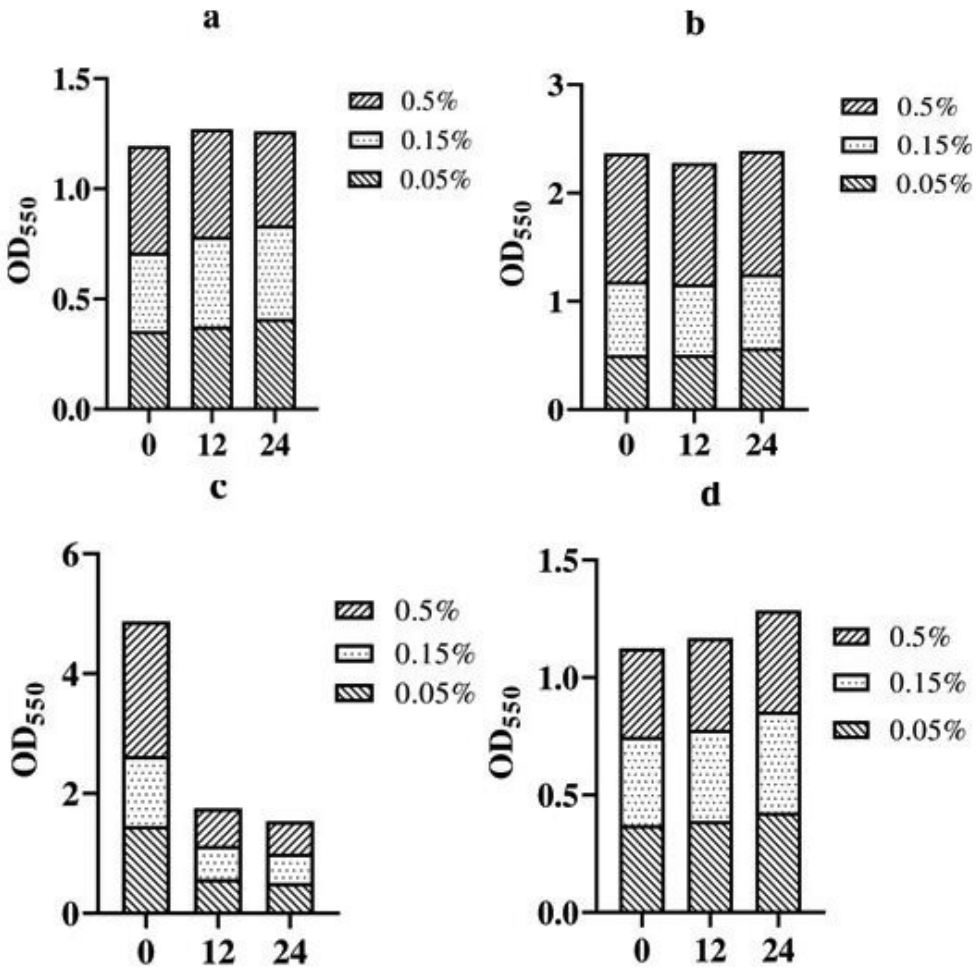

Figure 3. Effects of various concentrations of heavy metals on the growth of *B. cereus* BF2 over time.

According to Ghahfarokhi et al. [31] Ag showed good growth suppression on the *B. cereus* [31], which was dissimilar with our outcome. In case of Cu, inhibition of *Bacillus* spp. growth was reported in some experiments [32,33]. But a few studies indicated that the effect of Cu exposed no suppression on the *B. cereus* growth which was line with our investigation [34,35].

Conclusion

In this study, *B. cereus* BF2 has found significant survival at 0.3% bile salt and pH up to 8.0. The tolerance of *B. cereus* BF2 in culture medium supplemented with cadmium, copper, silver, and zinc was not found very distinguishable at qualitative assessment but diversified tolerance and viability response of the strain were observed in broth culture. Cd and Zn were found partial suppressive but could not stop the growth of *B. cereus* BF2. On the other hand, Cu and Ag were accumulated most significantly by *Bacillus* strain which was commensurate to our target of interest. To recapitulate, the results exhibited in this study indicate that *B. cereus* had phenomenal bioaccumulation and metal tolerant properties and it can clearly be manipulated regarding bioremediation purposes.

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Conflict of interest

The authors declared that they have no conflict of interest.

Authors' contribution

Salauddin Al Azad prepared the synopsis and conducted the total lab work, collected, and conserved the data obtained from the research. The rest four authors performed all statistical analysis and prepared the manuscript according to the suggestions and authorization of Salauddin Al Azad and reviewed the manuscript individually.

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