Chapter 8

Novel Microbial System Developed from Low-Level Radioactive Waste Treatment Plant for Environmental Sustenance

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Abstract

A packed bed bioreactor efficiently treated low-level radioactive waste for years with a retention time of 24 h using acetate as the sole carbon source. However, there was generation of dead biomass. This bioreactor biomass was used to develop a bacterial consortium, which could perform the function within 4 h while simultaneously accumulating nitrate and phosphate. The dead mass was negligible. Serial dilution technique was used to isolate the world’s first pure culture of a nitrate accumulating strain from this consortium. This isolate could simultaneously accumulate nitrate and phosphate from solution. Its ability to form biofilm helped develop a packed bed bioreactor system for waste water treatment, which could optimally remove 94.46% nitrate within 11 h in batch mode while 8 h in continuous mode from waste water starting from 275 ppm of nitrate. The conventional approach revealed the strain to be a member of genus *Bacillus* but showed distinct differences with the type strains. Further insilico analysis of the draft genome and the putative protein sequences using the bioinformatics tools revealed the strain to be a novel variant of genus *Bacillus*. The sequestered nitrate and phosphate within the cell were visualized through electron microscopy and explained the reason behind the ability of the isolate to accumulate 1.12
mg of phosphate and 1.3 gm of nitrate per gram of wet weight. Transcriptome analysis proposed the mechanism behind the accumulation of nitrate and phosphate in case of this novel bacterial isolate (MCC 0008). The strain with the sequestered nutrients work as biofertilizer for yield enhancement in case of mung bean while maintaining soil fertility post-cultivation.

**Keywords:** nitrate accumulation, packed bed biofilm reactor, Bacillus sp MCC 0008, insilico analysis, transcriptome analysis, radioactive effluent

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### 1. Introduction

All ore mining produce waste rock that in turn may produce acid mine drainage (AMD), due to the presence of sulfides. The waste generated is treated by physico-chemical means and is either stored in engineered containments or in open surface based on the nature of the effluent. Only limited information is available about effects of microbial processes used for similar purposes during large-scale operation. In addition, the mining itself and processing are often associated with a wide range of potential human health risks. Surface and underground mining generate a large volume of waste rock, which may contain only very little uranium but has fission products, for example, radium (radioactive) or lead (highly toxic) that is left behind as a waste. The second step is a process, known as the milling of the ore in which the rocks are crushed and ground. Chemical leaching follows and over 50% of uranium ore is obtained with classic mining methods. Water used in this process that cannot be recycled within a processing plant as well as excess water from a mine needs to be removed or treated to meet environmental requirements. The multistep process of recovery includes neutralization of the effluents, precipitating any metals, and reducing the uranium and radium content. [1–3]. This treatment depends upon the uranium recovery process, chemicals used, and contaminant ores. Water recovered may get recharged as groundwater or is either discharged or used for plant operations. Often this water needs further treatment before it could be reused or discharged for removal of contaminants. The multistep process begin with coagulating or precipitating heavy metals followed by neutralizing acids, or adjusting pH and then precipitating radium with barium chloride. The water treatment process is often followed by additional “clarification” or “polishing” steps using clarifiers, sand filters, and even reverse osmosis. The alternative option might be to use microbial bioremediation using sulfate-reducing bacteria [4].

The foremost source of waste generation occurs during nuclear fuel cycle operations that comprises of facilities to purify, convert, and enrich uranium from mining and milling and to manufacture fuel elements for nuclear reactor and gives rise to a variety of materials and product outputs [2, 3]. Enrichment of radioactive ore involves use of chemicals which lead to high levels of nitrate in the effluent.

The effluent generated cannot be discharged into the environment without treatment. The physicochemical treatment is expensive and economically not feasible during large-scale operation. Hence, biological options were sought. The problem in hand was to develop a microbial process, which could efficiently treat low-level radioactive waste containing nitrate...
generated from ore enrichment. Nitrate being a common pollutant in municipal as well as agricultural waste water, municipal sewage was passed through corrugated sheets of a packed bed reactor to develop a biofilm-based bioreactor that could treat low-level radioactive effluent within 24 h on a continuous basis [5] using acetate as the sole carbon source. However, dead mass was generated during the operation. The biomass was characterized [5] and further enrichment in nitrate broth (HiMedia M439) resulted in isolation of the fastest nitrate removing consortium. This consortium was further characterized to yield the world’s first nitrate accumulating pure culture [11] of a *Bacillus* sp. with immense application in terms of waste water treatment, plant growth promotion with seed quality enhancing ability. A combined approach of insilico and conventional analysis revealed the strain to be a novel species of genus *Bacillus*. In this chapter, we present the characterization of this novel isolate involved in bioremediation of soluble nitrate.

2. Consortium development and characterization

Nitrate removal from the medium by the bacteria was the primary step for selecting a consortium for nitrate removal. Either an assimilatory or a dissimilatory pathway results in nitrate removal from solution [6]. An alternative pathway for the nitrate removal was through nitrate accumulation, as evident in isolates from genus *Beggiatoa*, *Thiomargarita*, and *Thioploca*. Mussmann et al. [7] proposed a vacuolar nitrate accumulation mechanism linked to proton translocation in *Beggiatoa* sp. from marine origin. The bioreactor biomass treating low-level radioactive waste was selected as inoculum because it solely treated nitrate as a pollutant and would thus have stronger nitrate reducers/accumulators due to the constant exposure to nitrates in radioactive waste water. The consortium (BN7) developed in nitrate broth under aerobic condition reduced the nitrate within the range of 25–37°C temperature and pH 6–11. The consortium could form a biofilm with an optical density of 0.34. Conventionally, an optical density from 0.2 to 0.35 at 620 nm indicates a structured biofilm formation [8]. The biofilm formation was found to be strengthened (0.64) upon application of phytochemicals from

![Figure 1](http://dx.doi.org/10.5772/63323)

*Figure 1.* Picture of the biofilm-based packed bed bioreactor developed using the aerobic consortium BN7.
Mentha spicata. These results were validated by calculating the t value (25.23) for 19 df with a 95% confidence level using a two-sample one-tailed t-test with equal variance to yield a p value of 2.24025 E-16. The consortium was used to develop a packed bed biofilm bioreactor (Figure 1) for nitrate and phosphate removal in a continuous system.

The inoculum standardization indicated 10% of the parent culture as optimum for biofilm development. This consortium reduced 97.44% nitrate from the medium within 4 h (Figure 2) while simultaneously reducing 48.2% phosphate during incubation in a biofilm-based bioreactor. This consortium could reduce 500 ppm and 1000 ppm nitrate load within 7 and 5 h, respectively. Nitrate concentrations between 1500 and 4000 ppm could be reduced by 99% within 4 h (Figure 2), and 5000 ppm nitrate was reduced by 80.5% after treating for 11–12 h and 99.62% after 24 h. The correlation coefficient was −0.53173, which signifies no direct correlation between the initial nitrate load and bioreactor reduction for the range tested in this study. The above data show the aerobic consortium (BN7) to perform the fastest nitrate removal by a microbial system to the best of our knowledge.

On further analysis, this consortium was found to accumulate both nitrate and phosphate simultaneously (2.84 gm/gm wet weight for nitrate and 1.14 mg/gm wet weight for phosphate). Cd, Sr, and Ce inhibited the bacterial growth even at a concentration of 0.1 mM, whereas Co and Zn were inhibitory at 0.5 mM. For Cu, Fe, and Zn salts, lower concentrations had minimal impact on the nitrate reduction, and the reduction efficiency in the presence of Pb salts was at par with the control set. After 4 h of growth, 0.5 mM of Pb salts decreased the reduction efficiency by only 3%. Moreover, the nitrate reduction in the presence of Cu salts after 2 h was higher than for the control (37% in Cu-treated cells compared to 8.5% in control), which can be attributed to the presence of nirK, a Cu-dependent nitrate reductase gene. The two-sample one-tailed paired t-test for means was 21.73 for 2 df and at 95% confidence level; the corresponding p value was 0.001. Therefore, the nitrate reduction enhancement in the presence of
Cu was significant. However, the extent of this reduction decreased with increasing time due to the toxic effect of the metal on the microbes. For metals such as Fe, 0.1 mM and 0.5 mM inhibited the reduction by 4–6%, and similarly, 0.1 mM of Zn reduced the efficiency by 3–5%. The negative impact of metals on the reduction efficiency was significant for Co and Cr salts. For the Co treatment, the reduction after 4 h of growth dropped by 50% and 73% relative to the untreated cells for concentrations of 0.1 and 0.5 mM, respectively. Under similar growth conditions, decreasing the Cr salt concentration decreased the nitrate reduction by 30% while increasing the concentration decreased the reduction efficiency by 46% relative to the control cells. The Energy Dispersive X ray Fluorescence (EDXRF) analysis confirmed the metal accumulation in the biomass with the highest accumulation being for Pb (1200 ppb) followed by Cu (180 ppb), Cr (100 ppb), and Co (15 ppb). A single-factor ANOVA yielded a p value of 1.58 E–05 with an F of 13.90 and critical F of 2.70 for 22 df at a 95% confidence level. Hence, the difference in accumulation upon varied metal treatment was significant for BN7. A consortium capable of growing and accumulating such metals can be used for the bioremediation of nitrate and metal co-contaminants.

Preservation experiment revealed both subculture maintenance and glycerol stock storage at –80°C (two months storage) to be equally efficient with nitrate reduction efficiency of 94% and 92%, respectively, after 12 h of growth for BN7. Preserving the culture as a streak plate or stab reduced the efficiency to approximately 88%. The lyophilized form was less efficient relative to the other three storage methods. Thus, using a glycerol stock could be an efficient strategy for the long-term maintenance of the microbial consortium. At the molecular level, the BN7 harbored members which closely resembled Pseudomonas sp. (20%), Azoarcus sp. (31%), uncultured bacterium (46%) and Bacillus sp. (3%). The GenBank accession numbers were GU644465 to GU644489. A phylogenetic analysis was performed using the neighbor joining method (Figure 3) as stated above. The low Shannon diversity index value (0.39) confirms selective enrichment using a specific medium for nitrate reducers. An equitability index value (0.83) near 1 indicates that the different varieties observed were evenly distributed throughout the community. The genus Pseudomonas and Bacillus could be involved in the phosphate accumulation and nitrate reduction. Hence, a microbial consortium was developed which was acclimatized to low-level radioactive waste and could remove nitrate from it within 4 h of incubation at room temperature while generating little dead mass.

Figure 3. Phylogenetic tree depicting the position of one of the clones from BN7 constructed using the neighbor joining method.
3. Purification of nitrate accumulator and its characterization

Nitrate removal by denitrification and assimilation is well documented for bacterial species. Nitrate accumulation by bacterial genus *Beggiatoa*, *Thioploca*, and *Thiomargarita* [9, 10] is relatively a rare phenomenon. Moreover, all reports of such accumulation are in a mixed form or from environmental mixed samples [9, 10]. Before this study, no pure culture of a nitrate accumulator was reported. Serial dilution and streaking on nitrate agar plates were used to isolate the only pure culture of *Bacillus* sp. MCC0008 [11]. Among the pure strains isolated, MCC0008 was found to be a Gram-positive *Bacillus* (Figure 4a). Fatty Acid Methyl Ester (FAME) (0.733) as well as Phospholipid-Derived Fatty Acid (PLFA) analysis revealed similarity with *Bacillus cereus*. MCC0008 shows terminal endospore formation like *Bacillus subtilis* and unlike *Bacillus cereus* (which shows central endospore). Paraspor is absent, while the size is 1.85 μm by 0.899 μm. It has a generation time of 21.4 min and shows terminal endospore location. Table 1 shows the characteristics of the isolate.

![Figure 4a](attachment:image1.png)

**(a)** ESEM micrograph taken using ESEM, FEI QUANTA 200 MARK 2. **(b)** TEM micrograph taken using TEM, 120 kV, 5000× magnification. **(c)** Phosphate granules of 0.13–0.59 μm in the periphery of cells when grown in low phosphate concentration. **(d)** Phosphate accumulation throughout the cell when grown at high phosphate concentration.

| Enzyme production       | Catalase, oxidase, protease, amylase, lipase, DNAse positive, lecithinase negative |
|-------------------------|----------------------------------------------------------------------------------|
| Carbohydrate utilization| It utilizes dextrose, trehalose, esculin, glycerol, maltose                      |
| Plant growth promotion  | Phosphatase and ammonia production positive, indole acetic acid, hydroxymate      |
| traits                  | siderophore and hydrogen cyanide production negative                             |
| Antibiotic sensitivity  | Sensitive to ciprofloxacin, norfloxacin, cephadroxil, neomycin, gentamycin, doxycycline hydrochloride |
|                         | Resistant to metronidazole, rifampicin, ampicillin, trimethoprim, roxythromycin, cloxacin, ceftazidime |

*Table 1. Characteristics of Bacillus sp. MCC0008.*
The transmission electron micrographs clearly revealed the presence of vacuoles (Figure 4b) which has earlier been reported for nitrate accumulators. This indicates the possibility that the isolate is a nitrate accumulator. The nitrate accumulation study following sonication-based lysis of the harvested pellet and measurement of released nitrate from the intracellular cell free supernatant as per the method of Cataldo et al. [12] exhibited nitrate accumulation of up to 1278.66–1302.12 ppm/gm (0.021 M) of wet weight. It is less than the extent of accumulation reported for *Beggiatoa* but is the first pure isolate of a nitrate accumulator and also the first *Bacillus* reported to perform such function. The isolate accumulated 1115.25 μg/gm of wet weight of phosphate. The extent of phosphate accumulation was higher than that reported by type strain of *Acinetobacter baumanii*. The reason behind this enhanced efficiency was revealed by Transmission electron microscopy of whole cells which showed through and through accumulation of polyphosphate granules in this strain when grown in nitrate broth overnight with high phosphate concentration (Figure 4c and d).

The strain showed polysaccharide formation starting from the fourth hour that continued till the eighth hour. This property might provide the benefit of attachment to suitable surfaces to the strain. Active log-phase culture was used to determine whether the isolate could form biofilm according to the method of Martin et al. [8].

Different percentages (1%, 2%, 4%, 6%, 8%, 10%, 15%) of actively growing culture were inoculated in nitrate broth into small falcon containing identical number of plastic rachig rings. The performance in terms of nitrate and phosphate removal was checked for repeated recharges with sterile nitrate broth. The isolate showed good biofilm formation with 10% inoculum being the optimum. The biofilm formation showed saturation by eleventh hour. Optimum performance in terms of nitrate reduction was also observed in the eleventh hour (Figure 5). This optimization was further utilized for immobilization of the isolate in the reactor.

![Biofilm formation with time](image)

**Figure 5.** Extent of biofilm formation with time. The saturation was observed after 11 h of incubation.
Accompanied by this, the isolate’s ability for active biofilm formation was checked by assaying the supernatant in the tissue culture plate for nitrate and phosphate removal. By this, the time needed for biofilm formation along with optimal functioning in terms of nitrate and phosphate removal was determined to be 11 h for nitrate and 22 h for phosphate (Figure 6).

![Biofilm activity](image)

**Figure 6.** Optimization of time of incubation for biofilm performance.

Since the isolate grows as biofilm, it could be used for setting up of a biofilm-based bioreactor for continuous waste water treatment in terms of nitrate removal. However, a prerequisite for it was to design the minimal growth condition for the same. This would ensure that enrichment culture components would not be needed to run the process and in turn the influent would not add to the COD load of the effluent. Dextrose, glycerol, and citric acid were chosen to check the growth of MCC0008 in minimal condition. The isolate showed the best growth in glycerol, and hence, it was further utilized as the carbon source to determine the optimum percentage of carbon source for growth as well as performance. One percentage of glycerol showed the optimum growth as well as nitrate and phosphate removal under minimal condition. Hence, 1% of glycerol was standardized as the carbon source for the isolate for further studies in packed bed bioreactor.

The comparison of the isolate’s activity under different oxygen availability in the eighth hour after inoculation revealed that the isolate performed optimally in aerobic condition followed by anaerobic condition. Oxygen depletion in anaerobic state resulted in a decrease in activity. Highest amount of nitrate reduction and subsequent conversion to ammonia was also in aerobic state due to the assimilatory pathway. Substantial accumulation also occurred in
aerobic state so that the accumulated nitrate could be used as terminal electron acceptor in oxygen-depleted state.

In the 5 L suspended bioreactor, the strain grew exponentially up to 5 h with 65% denitrification and phosphate removal taking place within the fourth hour (Figure 7).

![Percent reduction in nitrate and phosphate using MCC0008 in 5l Bioreactor](image)

**Figure 7.** Percent reduction in nitrate and phosphate concentration with time using MCC0008 in 5 L suspended bioreactor.

4. **Immobilization and acclimatization in a packed bed bioreactor**

Fixed packed bed configuration has high surface area to volume ratio, thereby increasing the microbial density and improving the conditions necessary for nutrient removal. Biofilm-based reactors also have the advantage over other types of bioreactors with respect to ease of operation, high-density accumulation of microbe, resistance of the system to environmental stress [13] and do not require any additional measure to retain biomass in culture [14]. Rotating biological contractors (RBC), trickling filters and biofilm membrane bioreactor are some of the widely used biofilm-based bioreactor. Thus, in order to make the system more cost-effective along with better nutrient sequestration rate, the abilities of the isolate were further exploited. In order to exploit these biofilm forming, nitrate, and phosphate sequestration abilities, a reactor packed with suitable matrix with a fixed bed was developed. The bioreactor was designed of glass with steel mesh as immobilization matrix (Figure 8). The isolate could bind equally well to steel and plastic. The total capacity of the bioreactor was 9 L with a working volume of 5 L post-filling up with steel matrix up to sixty percent capacity. The steel mesh acted as the matrix for the formation of MCC0008 biofilm. Ports were designed at different heights of the bioreactor as shown in the Figure 8.
The graphical representation shows the initial acclimatization period for proper biofilm development. The initial rise and fall in the performance correlate well with the biofilm character of slough off and growth to achieve stability. It required about 30 loadings to attain stability. After the 39th loading, approximately full nitrate reduction was obtained in one hour only in nitrate broth which was retained for more than 90 days (Figure 9a).
After stable performance of the bioreactor in enriched media, next the performance of the reactor was monitored in minimal media (Figure 9b). This was done in order to acclimatize the reactor to minimal conditions before exposure to waste water. It contained 495ppm nitrate and 1% glycerol.

The biofilm was observed to be dense with thick layer of polysaccharide during environmental scanning electron microscopy (Figure 10).

Figure 9b. Performance in terms of nitrate removal in minimal medium with time.

Post-acclimatization of the biofilm to minimal media, non-radioactive wastewater was charged. The dynamics of nitrate removal in batch mode is reflected in Figure 11. Since the isolate is from a consortium acclimatized to radioactive waste water, it is expected to show similar performance with low-level radioactive waste.
Figure 11. Kinetics of nitrate removal from waste water in batch mode. (a) Nitrate reduction kinetics following non-linear curve fit (exponential). (b) Kinetics of remaining nitrate in the medium from 0 h (time of charging).

The equation, statistics, summary, and ANOVA for nitrate reduction kinetics (depicted in Figure 11a) are as follows:

\[ y = y_0 + A e^{(R_0 x)} \]

where \( y = \% \) Reduction, \( y_0 = \) initial nitrate concentration, \( x = \) time (in hours).

Statistics:

|                      | Reduction          |
|----------------------|--------------------|
| Number of points     | 12                 |
| Degrees of freedom   | 9                  |
| Reduced Chi square   | 7.39081            |
| Residual sum of squares | 66.51727     |
| Adj. R-square        | 0.99046            |
| Fit status           | Succeeded(100)     |

The equation, statistics, summary, and ANOVA for remaining nitrate in the medium with time (depicted in Figure 11b) are as follows:

\[ y = y_0 + A e^{(R_0 x)} \]

where \( y = \) Remaining nitrate in the medium, \( y_0 = \) initial nitrate concentration, \( x = \) time (in hours).

Complete nitrate removal from wastewater took place in 11 h. The longer retention time for waste water treatment as compared to that in minimal media in terms of nitrate removal may
be due to the presence of other contaminants to which the biofilm is sensitive. Multivariate analysis using response surface methodology revealed higher nitrate removal at higher initial concentration of nitrate with little effect of the flow rate (within the range tested) on the system performance (Figure 12).

Figure 12. The figure shows the response of nitrate concentration and flow rate on nitrate reduction in the bioreactor.

| Source              | Sum of Squares | df  | Mean Square | F     | p-value | Prob > F |
|---------------------|----------------|-----|-------------|-------|---------|----------|
| Model               | 279.17         | 9   | 31.02       | 17.36 | 0.0029  | Significant |
| A-Flow rate         | 14.96          | 1   | 14.96       | 8.37  | 0.0340  |
| B-Nitrate concentration | 76.14       | 1   | 76.14       | 42.61 | 0.0013  |
| C-Phosphate concentration | 1.25        | 1   | 1.25        | 0.70  | 0.4414  |
| AB                  | 0.93           | 1   | 0.93        | 0.52  | 0.5034  |
| AC                  | 4.56           | 1   | 4.56        | 2.55  | 0.1711  |
| BC                  | 0.39           | 1   | 0.39        | 0.22  | 0.6586  |
| A2                  | 0.42           | 1   | 0.42        | 0.24  | 0.6477  |
| B2                  | 12.37          | 1   | 12.37       | 6.92  | 0.0465  |
| C2                  | 14.61          | 1   | 14.61       | 8.18  | 0.0354  |
| Residual            | 8.93           | 5   | 1.79        |       |         |          |
| Lack of fit         | 7.84           | 1   | 7.84        | 28.59 | 0.0059  | Significant |
| Pure error          | 1.10           | 4   | 0.27        |       |         |          |
| Cor total           | 288.11         | 14  |             |       |         |          |
The final equations obtained through RSM-based optimization are as follows:

\[
\text{Nitrate reduction} = -81.1 + 424.3 \times \text{Flow rate} -0.049 \times \text{Nitrate concentration} + 4.74 \times \text{phosphate concentration} + 0.15 \times \text{Flow rate} \times \text{Nitrate concentration} - 6.04 \times \text{Flow rate} \times \text{Phosphate concentration} + 3.13e^{-004} \times \text{Nitrate concentration} \times \text{Phosphate concentration} - 292.25 \times \text{Flow rate}^2 - 5.002e^{-005} \times \text{Nitrate concentration}^2 - 0.018 \times \text{Phosphate concentration}^2
\]

The packed bed bioreactor system could treat waste water optimally removing 94.46% nitrate within 11 h in batch mode while 8 h in continuous mode from waste water containing 275 ppm of nitrate at 0.63 L/h flow rate.

5. Application as biofertilizer

Singh et al. [15] conducted experiments using *Advenella species* (PB-05, PB-06, and PB-10) and *Cellulosimicrobium* sp. PB-09 to analyze the IAA production, HCN production, ammonia production, and phosphate solubilization and correlated the results to the isolates’ capability to promote plant growth. For them the isolates positively affected all characteristics except HCN production [15]. Since the isolate MCC0008 could accumulate both nitrate and phosphate simultaneously and also produce phosphatase, its effect on plant growth promotion was checked in case of mung bean (Vigna radiate var Samrat). Table 2 representing the germination
percentage, germination index, and vigor index for Mung bean (Vigna radiata) seeds with and without treatment with isolate (soil and seed application) revealed better germination upon soil application. It was expected since the isolate produces plant growth hormones.

Table 2. The table shows the germination parameters in case of mung bean upon application of MCC0008.

| Sample          | Control | MCC0008 (coated) | MCC0008 (soil) |
|-----------------|---------|------------------|---------------|
| Germination percentage | 74.074  | 83.333           | 87.037        |
| Germination index          | 39.772 ± 9.39 | 62.298 ± 12.234 | 75.313 ± 9.44 |
| Vigor index                   | 1639.056   | 2390.688         | 2006.801      |

Soil application gave better result, and so further experiments were conducted by sowing soaked seeds, followed by soil application of the isolate. The germination in the presence of antifungal agent (Saaf) was better upon application of the isolate to soil.

Table 3. Chance in elemental content of seed grown without fertilizer (control), with chemical fertilizer and with biofertilizer.

| Elements | MCC0008 | Chemical |
|----------|---------|----------|
| Zn       | 16.04   | −7.99    |
| Fe       | 2.84    | −7.20    |
| Mn       | 14.49   | 7.08     |
| Cu       | 25.41   | 8.97     |
| P        | 12.82   | −66.60   |
| K        | 4.39    | −19.16   |
| S        | 12.57   | −26.24   |
| Ca       | 5.59    | −12.59   |

The control was taken as reference and that for biofertilizer and chemical fertilizer was calculated accordingly.

Pot trial and field trial were carried out. For field trial, randomized block design with four replicates was carried out. The sowing was done in the north–south orientation. The seeds’ post-germination was subjected to thinning such that each 1 m² area contained a total of 40 plants (4 rows of 10 plants each). The inoculum for the germination trial was $4.2 \times 10^6$ cells per 125 gm soil in a thermo coal glass/germination tray, $1.39 \times 10^7$ cells per 8 kgs soil in each pot and $3.68 \times 10^9$ cells per 1 m² plot for field trial. The yield per hectare of land was calculated for the consortium when compared with control (without fertilizer) and chemical fertilizer application. The yield per hectare for control, MCC0008 application, and chemical fertilizer application was 1277.5 kg, 1974.5 kg, and 1685 kg, respectively. The elemental content improved post-application as compared to control as measured through EDXRF analysis.
(Table 3). This shows that not only the yield improves as compared to chemical fertilizer but also the elemental content was better as compared to control as well as chemical treatment in MCC0008-treated seeds. The data revealed that upon treatment with MCC0008, there was desirable change in the nutritional quality parameters. There was increase in energy value (4.3%), total carbohydrate (4.5%), total sugar (0%), total dietary fiber (4.5%), protein (4.9%) content while a decrease in moisture content (23.4%), total ash (6.4%), and crude fat (7.5%). The decrease in moisture would ensure better storage of the grains, decrease in ash content means less non-utilizable component, while decrease in fat improves the quality further.

Figure 13. Yield enhancement of mung bean in the presence of biofertilizer with and without gamma irradiation.

According to the previous reports, gamma irradiation of seeds brings about faster germination [16–18]. This is due to increased levels of transcription. The antinutrient as well as elemental levels following irradiation (presowing) is also reported to be lower. Thus, a combined effect of low-dose gamma irradiation of mung bean seeds along with biofertilizer application was tested. The effect of combined application of low-dose gamma irradiation (2.6 Gray and 5 Gray) on germination, yield enhancement, and elemental content of mung bean seeds were tested. The cell structure and viability of the irradiated seeds were studied following ESEM analysis and microtomy using standard techniques. There was mild improvement in germination following irradiation at 5 Gray while significant yield enhancements in irradiated seeds as shown in the Figure 13.

Figure 14a. ESEM image of control and irradiated seeds showing part of the seed coat and hilum.
In order to explore the reason behind improved germination, detailed analysis of seed structure and hilum morphology was carried out using ESEM as shown in Figure 14a and b.

**Figure 14b.** ESEM analysis seed coat and the hilum of un-irradiated and irradiated seeds.

However, this depth of analysis could reveal just dehydration and nothing beyond that. Dehydration is expected to delay germination, while here we observe faster germination. Hence, there must be some other phenomenon which is induced during irradiation. Since germination is initiated through hilum and it is the point of contact for imbibition of water, further analysis with conventional microtomy was carried out. It revealed loosening of the compact arrangement of protein sheets with starch granules upon irradiation (Figure 15). Since irradiation might inactivate the germplasm hence viability staining was carried out for the
same set, it was revealed that the vitality of the seeds was maintained for the irradiated seeds within the range tested. Hence, low-dose gamma irradiation does not destroy the seed but makes the hilum loose to enable better uptake of water and nutrients and hence faster germination and enhanced yield. Further analysis at the transcription level will be required for better understanding of the phenomenon.

Figure 15. Microtomy images of hilum at 40× magnification of Dewinter Trinocular Microscope (New Crown) showing disintegration of compact protein sheets with irradiation.

The application of this strain as biofertilizer to enhance yield while maintaining nutritional quality of the grain and soil fertility has been filed as patent application in India [19]. To protect the intellectual property associated with this discovery, a PCT has also been filed [19].

6. Bioinformatics-based strain identification

The genus “Bacillus” has a long history of importance, both from an economic point of view and as a source of experimental microorganisms. Bacteria of the genus *Acinetobacter* were originally thought to be the major PAOs (polyphosphate accumulating organism). The pure isolate of nitrate accumulating *Bacillus* sp. MCC0008 showed potential for waste water treatment as well as biofertilizer application, hence of immense commercial importance. Knowing the identity of the strain becomes essential for better understanding of the system. This study was undertaken to decipher its species identity as per standard procedure [20] while exploring its underlying phenomenon of nitrate and phosphate accumulation. ANI (Average Nucleotide Identity) was calculated using ANI calculator for this strain with respect to the type strains of *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis*. The ANI calculator estimates the average nucleotide identity using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) between two genomic datasets [21]. Inter-genomic distances between this strain and its closest neighbors were determined using Version 2.0 of the DSMZ Genome-To-Genome Distance calculator, an *insilico* version of DNA-DNA hybridization [20]. The draft genome of each isolate was compared to the genome sequence of the type strains of *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis* using dot plot analysis through a genomic similarity search tool, YASS [20, 22], to understand the similarities between the isolates over the length of their genomes. The contigs were uploaded to the Rapid Annotation using
Subsystems Technology (RAST) server, which is a fully automated service for annotating bacterial and archaeal genomes and provides high-quality annotation for these genomes across the phylogenetic tree. The annotated genomes in the seed viewer depicted the metabolic patterns for the strain and the four reference Bacillus strains. The gene arrangements on each chromosomal segment were compared for the strain with that of the other Bacillus sp. for phosphate metabolism as per earlier studies [20]. Furthermore, metabolic pathway reconstruction was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database through RAST. The genomes were compared in terms of the number of genes involved in different metabolic pathways and in phosphate metabolism as compared with the type strains.

Figure 16. Genomic comparison of the draft genome of MCC0008 (also named WBUNB001) with other members of Bacillus species. (A) Pie chart of the data generated following blast analysis of the contigs revealing maximum similarity with different organisms. Maximum similarity of major portion of the contigs is with Bacillus cereus. (B) Represents the comparison of the genome of MCC0008 with Bacillus thuringiensis. The graphs depicted gene transfer within the genome (GC content) while the GC skew data which should be 50% positive and 50% negative under ideal condition showed 45–50% +ve with Bacillus thuringiensis. (C) Represents the comparison of the genome of MCC0008 with Bacillus anthracis. The graphs depicted gene transfer within the genome (GC content) while the GC skew data which should be 50% positive and 50% negative under ideal condition showed about 30%+ve with Bacillus anthracis. The gap in the genome sequence was also revealed through this analysis. This analysis also revealed maximum identity with Bacillus cereus at the nucleotide level. (D) Mauve analysis to determine genome rearrangement when compared with Bacillus anthracis. There is extensive rearrangement in all three cases emphasizing the isolate to be a novel species different from these three type strains of Bacillus sp.
The phylogenetic analysis of the novel strain was done with its closest neighbors using the 16SrRNA sequence as well as using seven housekeeping genes, namely RNA polymerase B (rpoB), gyrase B subunit (gyrB), pyruvate carboxylase A (pycA), malate dehydrogenase (mdh), rod shape determining protein (MreB), DNA mismatch repair protein (MutS), and transcription regulator (plcR). The software used was MEGA (Molecular Evolutionary Genetics Analysis) Version 6.0. MEGA is an integrated tool which is used for constructing sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species.

The draft genome sequence of Bacillus sp. MCC0008 had a total number of assembled reads of 1,740,538, with 331 contigs (43 × coverage), in which 315 were large with 35.1% G + C content. A total of 307Mb were sequenced with 202Mb having quality values >20 [11]. Average nucleotide identity and phylogenetic analysis revealed that Bacillus sp. MCC0008 is closest to Bacillus anthracis while FAME (Fatty Acid Methyl Esters), PLFA (Phospholipid-Derived Fatty Acids), BLAST (Basic Local Alignment Search Tool), Dot plot and BRIG (BLAST Ring Image Generator) analysis and partial 16SrDNA revealed maximum identity with Bacillus cereus. However, MAUVE analysis performed on the draft genome of MCC0008 (GenBank Accession Number: ANAU00000000) with the type strains of Bacillus cereus, Bacillus thuringiensis, and Bacillus anthracis revealed extensive genomic rearrangements while RAST analysis revealed 40% subsystem coverage whereas remaining 60% did not have identity with any known sequence stretch. From the combined interpretation, it is apparent that the strain under investigation is novel species of genus Bacillus (Figure 16).

| House-keeping genes                      | Closest neighbor                                      |
|------------------------------------------|-------------------------------------------------------|
| DNA gyrase subunit B                    | Bacillus anthracis str Ames and Bacillus anthracis str Sterne |
| DNA-directed RNA polymerase beta subunit | Bacillus thuringiensis serovar konkukian str 97-27   |
| Malate dehydrogenase                     | Bacillus anthracis str A1055                          |
| DNA mismatch repair protein mutS         | Bacillus anthracis str Ames and Bacillus anthracis str Sterne |
| Phosphatidylinositol specific phospholipase C | All the strains of Bacillus anthracis                 |
| Rod shape determining protein MreB       | Bacillus anthracis str Ames and Bacillus anthracis str Sterne |
| Pyruvate carboxyl transferase            | Bacillus thuringiensis str. Al Hakam                 |
| Partial 16S rRNA                         | Bacillus cereus                                       |

Table 4. Closed neighbor of MCC0008 in case of the house-keeping genes.

DNA–DNA hybridization which calculate the inter-genomic distances between the strains with the score of >70% indicates the same species. The strain was compared with the type strains of Bacillus anthracis (Ba), Bacillus thuringiensis (Bt), and Bacillus cereus (Bc) revealing a value of 81.8 ± 2.72%, 79 ± 2.82%, and 61.3 ± 2.83% respectively. Hence Bacillus sp. MCC0008...
was closest to *Bacillus anthracis* (Ba), followed by *Bacillus thuringeinsis* and had the least identity with *Bacillus cereus*. The phylogenetic analysis of the different housekeeping genes at the nucleotide sequence level showed similarity with different species of genus *Bacillus* as revealed in Table 4 indicating it to be a novel species of genus *Bacillus*.

### 7. Phylogenetic analysis of putative protein

The nucleotide sequence stretches: ANAU01000001, ANAU01000016, ANAU01000020, ANAU01000033, ANAU01000036, ANAU01000046, ANAU01000052, ANAU01000062, and ANAU010000274—each containing several genes—from the draft genome of MCC0008 [11] were translated in MEGA6 [23] using the standard genetic code. The protein sequences generated from these stretches were submitted to HAMAP [24], Interproscan [25, 26], EMBL-Fasta [26, 27], Prositecan [26, 27], and NPSA blast [28] for predicting their functions. The largest amino acid sequence stretch derived from ANAU01000036 was divided into parts, and the protein blast search of NCBI [29] was used to decipher the function of its individual proteins. The consensus predictions from the tools used, were selected for further detailed analysis. Each of the prediction was verified by scanning the proteins for function specific sequence signatures, using the Scanprosite [30, 31] tool. Alternatively, conserved patterns were identified from the HAMAP seed alignment [24] and uniprot protein cluster—UniRef [32] of the said functional protein category.

| Nucleotide sequence stretch of MCC0008 | Putative protein | Closest species | Prosite entry | HAMAP entry | UniRef entry | Sequence motif in MCC0008 |
|---------------------------------------|-----------------|----------------|---------------|-------------|---------------|--------------------------|
| ANAU01000001                          | Malate synthase | *Bacillus cereus* | PS00510       |              |               | KDHSAGLNCGRWDYIF          |
| ANAU01000001                          | NAD kinase      | *Bacillus anthracis* | MF_00361     |              |               | GGDG                     |
| ANAU01000001                          | FabH            | *Bacillus cereus* | MF_01815     |              |               | AACAGF                   |
| ANAU01000001                          | ATP dependent helicase | *Bacillus cereus* | MF_01452     |              |               | LIA                      |
| ANAU01000001                          | Peptide ABC transporter permease | *Bacillus anthracis* | PS00928     |              |               | TRVSLYIALLAAIDLVGAYGGISAF |
| ANAU01000001                          | spx transcription regulator | *Bacillus thuringiensis* | MF_01132 |              |               | IDEKRLQVGY, SCTSC         |
| ANAU01000016                          | Quinone oxidoreductase | *Bacillus cereus* | PS01162      |              |               | VLIHAAGGIGTT             |
| ANAU01000020                          | Zinc containing alcohol dehydrogenase | *Bacillus thuringiensis* | PS00059 |              |               | GHEFSGEV                 |
| ANAU01000020                          | Transaldolase   | *Bacillus cereus* | PS01054      |              |               | GVTNPSLV                 |
| ANAU01000020                          | Phosphate uptake ABC transporter permease | *Bacillus anthracis* | PS00928 |              |               | RLCIETMASLPSIVVGFLVFTMGTG |
| Nucleotide sequence stretch of MCC0008 | Putative protein | Closest species | Prosite entry | HAMAP entry | UniRef entry | Sequence motif in MCC0008 |
|--------------------------------------|------------------|----------------|--------------|-------------|-------------|--------------------------|
| ANAU01000020 FAD dependent oxidoreductase | Bacillus cereus | P00862 | | | | IRVVGSGH |
| ANAU01000020 GerLA | Bacillus anthracis | UniRef50_Q93N70 | | | | FAMYVALSVYHCGLI |
| ANAU01000020 GerLB | Bacillus cereus | UniRef50_Q93N69 | | | | GTYLA |
| ANAU01000033 Phosphoglycerate kinase | Bacillus cereus | PS00111 | | | | RVDFNVP |
| ANAU01000033 Uvr domain A | Bacillus cereus | PS0151 | | | | EKTIKAEMKAFAAKD |
| ANAU01000033 Uvr domain B | Bacillus cereus | PS0151 | | | | EKTIKAEMKAFAAKD |
| ANAU01000033 Central glycolytic genes regulator | Bacillus thuringiensis | UniRef90_A0RKS8 | | | | SASLGMT |
| ANAU01000033 Murein hydrolase export regulator | Bacillus anthracis | UniRef50_Q6HR39 | | | | TVAJASD |
| ANAU01000033 Transcription regulator WhiA | Bacillus anthracis | UniRef50_Q66975 | | | | TLHELDMV |
| ANAU01000033 Autotransporter | Bacillus cereus | UniRef90_B7HG2 | | | | LKREV |
| ANAU01000036 Acetyl ornithine deacetylase | Bacillus cereus | PS0072 | | | | ALTEPNAGSDALS |
| ANAU01000036 Acyl co-A dehydrogenase | Bacillus anthracis | PS00600 | MF_00832 | | | YDQR |
| ANAU01000036 Alpha beta hydrolase | Bacillus cereus | PS01103 | | | | MAATCVRVPVS |
| ANAU01000036 Aminotransferase classIII | Bacillus thuringiensis | UniRef90_A0RKS8 | | | | SASLGMT |
| ANAU01000036 ATPase AAA | Bacillus cereus | UniRef50_A0IAW6 | NFNEN | | | |
| ANAU01000036 Chloramphenicol acetyltransferase | Bacillus cereus | UniRef50_A0REA1 | | | | GETMG |
| ANAU01000036 Chlolesterolytic hydrolase | Bacillus cereus | UniRef50_Q61H11 | | | | GVNEH |
| ANAU01000036 Citrate synthase | Bacillus thuringiensis | PS00480 | | | | GFGHRVY |
| ANAU01000036 Cold shock protein | Bacillus anthracis | UniRef50_Q45096 | | | | NLFA |
| ANAU01000036 D-alanine D-alanine carboxypeptidase | Bacillus cereus | UniRef50_Q6HP8 | | | | SYAAP |
| ANAU01000036 Diguanylate cyclase | Bacillus cereus | UniRef50_A0IR9R1 | | | | NITL |
| ANAU01000036 DNA binding protein | Bacillus thuringiensis | PS0943 | | | | LKTIREEKLSLEKVSQUTY |
| ANAU01000036 Glucokinase | Bacillus cereus | UniRef90_Q738U1 | | | | YQLSRYYV |

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Table 5. Comparison of contigs of MCC0008 with closest neighbor at the putative protein sequence level.

Hence from the combined interpretation it is concluded that due to extensive genomic rearrangement, *Bacillus* sp. MCC0008 has emerged to be a novel species.

The members of the Genus *Bacillus* comprising of *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis* [33–35], which share high degree of sequence similarity with MCC0008, were chosen for the functional annotation and the phylogenetic study of MCC0008. The protein sequences of the above group members, having the functions as predicted in MCC0008, were retrieved from the protein database of NCBI [36] as available. The sequences which could be acquired were aligned in MEGA6 [23] with the corresponding translated nucleotide stretches of MCC0008, using the clustalW program [37]. The protein weight matrix was set to BLOSUM [38]. The prosite motifs/conserved patterns from HAMAP seed alignment/UniRef, pertaining to the relevant function, were searched in the alignments.

The consensus predictions for the translated nucleotide sequence stretches of the draft genome of MCC0008 are summarized in Table 5. The putative proteins showed sequence specific characteristics of the predicted functions, as validated through sequence motifs in the prosite database/HAMAP family profile/UniRef. The sequence alignments of the MCC0008 proteins

| Nucleotide sequence stretch of MCC0008 | Putative protein | Closest species | Prosite entry | HAMAP entry | UniRef entry | Sequence motif in MCC0008 |
|---------------------------------------|------------------|-----------------|---------------|-------------|--------------|--------------------------|
| ANAU01000036 Membrane protein         | *Bacillus*       | *Bacillus*      | UniRef50_C3BJ03 |             |              | LGITV                    |
| ANAU01000036 MFS transporter          | *Bacillus*       | *Bacillus*      | PS0850        |             |              | MIRILAVAFFVGLDSLVVAP     |
| ANAU01000036 Multidrug ABC transporter| *Bacillus*       | *Bacillus*      | PS0893        |             |              | GPTGSKKTHINLTFREYD       |
| ANAU01000036 NADH ubiquinone oxidoreductase | *Bacillus* | *Bacillus* | UniRef90_Q81K10 |             |              | ARGVYANA                 |
| ANAU01000036 Serine threonine protein kinase | *Bacillus* | *Bacillus* | PS0111        |             |              | ICMSYGTVYVV              |
| ANAU01000036 Threonyl tRNA synthetase | *Bacillus*       | *Bacillus*      | MF_0184       |             |              | GFYYD, GAYWRGD           |
| ANAU01000046 Feril                     | *Bacillus*       | *Bacillus*      | UniRef90_Q64HFP8 |             |              | NTYYKKHELRAVW           |
| ANAU01000052 Nitrile/Nitrate response regulatory protein | *Bacillus* | *Bacillus* | PS0110        |             |              | SVLVVDHVAVGLGKALIEKYDDMVVEVVDST |
| ANAU01000052 ABC transporter          | *Bacillus*       | *Bacillus*      | PS0893        |             |              | ILKQGELTGVVGKTSKTTLVRQ   |
| ANAU01000062 Non homologous End joining protein Ku | *Bacillus* | *Bacillus* | MF_01875      |             |              | WKG                      |
| ANAU01000062 Spore coat coatA         | *Bacillus*       | *Bacillus*      | UniRef50_Q45536 |             |              | HISPQDPFPQGKYY           |
| ANAU010000274 Hypothetical protein    |                  |                 |               |             |              |                          |
with the corresponding proteins of the Genus Bacillus and the presence of signatures from Prosite/HAMAP/UniRef therein brought out the sequence motifs of the group and the MCC0008 strain. The database entries along with the corresponding exact motif in MCC0008 are tabulated in Table 5 again. The high degree of sequence similarity amongst MCC0008 and the members of Genus Bacillus resulted in these sharing the same protein sequence motif, with a few exceptions of diverging sequences of Bacillus sp. These hint that the isolated strain being reported could belong to the Genus Bacillus but not any of these known species. The phylogenetic trees computed for the different proteins show that in most of the cases, Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis gets clubbed with MCC0008, with Bacillus mycoides, Bacillus pseudomycoides, and Bacillus weihenstephanensis being clad out. The study indicates that MCC0008 is closest to cereus, anthracis, and thuringiensis. Further, Bacillus cereus emerges nearest to MCC0008 for ATP-dependent helicase, FabH, Malate synthase, Quinone oxidoreductase, FAD-dependent oxidoreductase, Transaldoase, GerLB, Uvr system domain A, Autotransporter, D-alanyl D-alanine carboxypeptidase, Glucokinase, NADH ubiquinone oxidoreductase, Membrane protein, Acetyl ornithine deacetylase, ATPase AAA, Serine threonine protein kinase, Diguanylate cyclase, Threonyl tRNA synthetase, Alpha beta hydrolase, Choloramphenicol acetyltransferase, MFS transporter. Aspartate semialdehyde dehydrogenase, Choloylglycine hydrolase, Multidrug ABC transporter ATP binding protein, Fni, ABC transporter, Nitrite/nitrate response regulatory protein, End joining protein ku, spore coat protein CotJA. Bacillus anthracis on the other hand appears closest to MCC0008 for peptide ABC transporter permease, NAD kinase, Phosphate uptake ABC transporter, Phosphoglycerate kinase, GerLA, Uvr system domain B, Murein hydrolase export regulator, sporulation regulator WhiA, Acyl co-A dehydrogenase, Cold shock protein. Spx transcription regulator, Zn-containing alcohol dehydrogenase, Central glycolytic genes regulator, Amino-transferase class III, Citrate synthase, and DNA-binding protein show MCC0008 getting clubbed with Bacillus thurgiensis. The picture that emerges here is that the strain in question seems to be a novel species mostly toward Bacillus cereus, with traces of Bacillus anthracis and a dash of Bacillus thurgiensis. The probable novel strain MCC0008 which shares traits from Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis could have emerged from genetic rearrangements between these species of the Bacillus group. Bacillus anthracis which is not reported to be a phosphate accumulator appears nearest to MCC0008 for phosphate uptake ABC transporter permease and phosphoglycerate kinase. Bacillus cereus is in closest proximity to MCC0008 for the nitrite/nitrate response regulatory protein. It appears from these observations that the genetic components from cereus, anthracis, and thuringiensis have given rise to this novel strain which has acquired the unique property of phosphate and nitrate accumulation.

8. Transcriptome analysis (BioProject PRJNA222597)

From transcriptome analysis (Figure 17 and Table 6), it is concluded that there is significant upregulation of sporulation genes, which can be due to the accumulation of poly-P in the bacterial cells [39]. The sporulation of Bacillus species initiates with the asymmetric division of
cellular compartment into two parts: the mother cell and the forespore. In the model organism *B. subtilis* (Bs), this process is temporally and spatially regulated by a set of sigma factors of RNA polymerase: the main vegetative sigma factor SigA and SigH in the pre-asymmetric division cell; SigE and SigK in the mother cell; and SigF and SigG in the forespore. The DNA-binding protein Spo0A is the master regulator for entry into sporulation in *B. subtilis* [40]. Further there is significant upregulation of serine protein kinase which also play a role in sporulation [41] and also there is upregulation of histidine kinase which also play a significant

![Heat map](http://dx.doi.org/10.5772/63323)  

**Figure 17.** Heat map of top 100 differentially expressed transcript contigs in control and treated samples.
role in sporulation. The initiation of sporulation in *Bacillus subtilis* and most likely in aerobic *Bacillus* species in general is controlled by the phosphorelay signal transduction system [42]. The ultimate goal of the phosphorelay is to activate by phosphorylation the Spo0A transcription factor, which represses certain genes and promotes the transcription of a large number of genes for stationary-phase functions as well as sporulation [40, 43]. The signals that initiate the phosphorelay reactions are recognized and interpreted by several sensor histidine kinases [44–47]. The initiation of sporulation in *Bacillus subtilis* is controlled. *Bacillus* sp. MCC0008 synthesizes poly-p granules as revealed from the significant upregulation of phasin and also through polyphosphate staining and TEM analysis. Phosphorus (P) is an essential element for all cells as it is a component of, for example, DNA, RNA, and membrane lipids. The common phosphorus source is inorganic phosphate (Pi), which is taken up by bacteria either via secondary transporters or via ATP-driven ABC transporters. Extracellular phosphate esters can serve as an alternative P source. Phosphate esters are hydrolyzed by bacterial phosphatases and the resulting Pi imported into the cells. In addition, some bacteria utilize specific uptake systems for the transport of sn-glycerol-3-phosphate as organophosphate. The intracellular Pi is assimilated into cellular metabolites by reactions such as F1F0-ATP synthase or glyceraldehyde-3-phosphate dehydrogenase. Moreover, polyphosphate can be formed as a readily available intracellular Pi source. From the transcriptome analysis, it is revealed that there is downregulation of glyceraldehyde-3-phosphate dehydrogenase; hence, there is no assimilation of phosphate in the form of poly-P.

For nitrate accumulation, it is hypothesized that the nitrate accumulation occurs due to electrochemical gradient (Δp) [48]. In plants, typically vacuolar-type H+ATPases and H+pyrophosphatases (HPPases) catalyze a proton translocation over endomembranes to generate a Δp for solute transport and likely also nitrate transport [49]. Vacuolar-type ATPases also occur in plasma membranes of some Archaea, but they are rarely encountered in Bacteria [50, 51]. A vacuolar Hþ-pyrophosphatase (hppA) and an uncommon Ca2+ translocating ATPase, may also contribute to generation of a Δp/ΔPh [52]. From transcriptome analysis it is revealed that there is 3.95-fold change in cation-transporting ATPase, which can be responsible for electrochemical gradient and nitrate accumulation.

| Genes   | Protein encoded                        | Fold Change | log2Fold Change | Function                                                                 |
|---------|---------------------------------------|-------------|-----------------|--------------------------------------------------------------------------|
| BCK_14255 | Stage III sporulation protein AH      | 12.53       | 3.65            | Involved in forespore engulfment                                          |
| BCK_05395 | Serine protein kinase                  | 11.43       | 3.51            | Kinase enzyme that phosphorylates the OH group of serine                  |
| BCK_14250 | Stage III sporulation protein AG      | 10.31       | 3.37            | Sporulation resulting in formation of a cellular spore                   |
| BCK_17375 | Hypothetical                          | 12.84       | 3.68            | Unknown                                                                  |
| Genes    | Protein encoded         | Fold Change | log2Fold Change | Function                                                                 |
|----------|-------------------------|-------------|-----------------|--------------------------------------------------------------------------|
| BCK_14245| Stage III sporulation protein AF | 8.94        | 3.16            | Leading to endospore formation                                            |
| BCK_08950| Stage II sporulation protein  | 8.80        | 3.14            | Sporulation resulting in formation of cellular spore                      |
| BCK_17370| Uncharacterized protein   | 8.08        | 3.01            | Unknown                                                                  |
| BCK_23315| Uncharacterized protein   | 9.89        | 3.31            | Unknown                                                                  |
| BCK_12895| Stage VI sporulation protein D | 6.18        | 2.63            | Required for assembly of a normal spore coat. May be a component of the innermost layer of the spore coat that aids in its adherence to the prespore. |
| BCK_02050| 2-oxoglutarate dehydrogenase E1 component | 5.89        | 2.56            | The 2-oxoglutarate dehydrogenase complex catalyzes the overall conversion of 2-oxoglutarate to succinyl-CoA and CO₂. It contains multiple copies of three enzymatic components: 2-oxoglutarate dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and lipoamide dehydrogenase (E3). |
| BCK_02290| Uncharacterized protein   | 5.81        | 2.54            | Unknown                                                                  |
| BCK_08430| Uncharacterized protein   | 5.84        | 2.55            | Chromatin binding                                                        |
| BCK_17365| Hypothetical protein      | 5.67        | 2.50            | Unknown                                                                  |
| BCK_02225| Spore coat protein Z      | 5.92        | 2.57            | Sporulation resulting in formation of a cellular spore                   |
| BCK_04810| Ribose import ATP-binding protein RbsA | 5.07        | 2.34            | Part of the ABC transporter complex RbsABCD involved in ribose import. Responsible for energy coupling to the transport system. |
| BCK_03255| Group-specific protein    | 6.24        | 2.64            | Catalyses the phosphorylation of incoming sugar substrates concomitant with their translocation across |
| Genes     | Protein encoded          | Fold Change | log2Fold Change | Function                                      |
|-----------|--------------------------|-------------|-----------------|-----------------------------------------------|
| BCK_03260 | Histidine kinase         | 4.02        | 2.01            | Phosphorelay sensor kinase activity           |
| BCK_04960 | Amino acid ABC transporter ATP-binding protein | 3.38 | 1.76 | –                                             |

Table 6. Table showing fold change in expression of different genes during transcriptome analysis of MCC0008.

9. Conclusion

The isolate MCC0008 is an extracellular protease, amylase, lipase, catalase, oxidase, phosphatase, and DNAse secreting strain which can form well-structured biofilm. It was isolated from a consortium developed from low-level radioactive waste treatment plant biomass. The strain is a novel species of genus *Bacillus* which falls within the group of *Bacillus cereus*. It could sequester nitrate within one hour from nitrate broth while took 11 hours to do the same from waste water under minimal condition in batch mode. This could be due to the antagonistic effect of the natural microflora of waste water or non-biological inhibitors. The well-developed biofilm ensured sustained performance of the system. The isolate during soil application retains phosphate and nitrate in the root zone ensuring better access to the plants. This was the reason behind approximately twofold and 1.2-fold yield enhancement as compared to no fertilizer and chemical fertilizer application respectively. Hence, microbial isolate from the low-level waste water treatment plant could help sequester essential plant growth nutrients from waste water. This would help reuse these nutrients while purify the water which in turn can be reused for agriculture and aquaculture, hence preventing wastage of potable water for non-potable application. This solution leads to environmental protection (prevention of eutrophication) and sustenance (organic farming).

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