Cellulosimicrobium funkei: A Novel Bacterium in Potassium Solubilization from Soil in Bangalore

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Abstract— *Potassium (K) is a very essential element needed by plants for healthy growth and good yield. Most soils have abundance of potassium underneath in rock as insoluble forms that are unavailable for plant use. This research was carried out to join in the search to unearth microorganisms from the rhizosphere soil that are able to act on the mineral containing substances, solubilizing them to release the needed soluble form of the potassium for plant use. An isolate, which was characterized and identified to be Cellulosimicrobium funkei, showed significant solubilization on feldspar (a potassium containing compound) supplemented media. It is novel for potassium solubilization. The amount of potassium released by the isolate in comparison to reference cultures varied but favourably compared with the reference cultures. In glucose amended broth, solubilization was: Cellulosimicrobium funkei 7.04mg/l, Enterobacter hormaechei 7.15 mg/l and 6.91mg/l for Aspergillus terreus. Urea supplemented broth: Cellulosimicrobium funkei 5.45mg/l, Enterobacter hormaechei 5.38mg/l and Aspergillus terreus 6.33mg/l. KCl supplemented broth: Cellulosimicrobium funkei 10.23mg/l, Enterobacter hormaechei 8.05mg/l and Aspergillus terreus 9.11mg/l. For temperature, the cultures solubilized best at these respective temperatures: Cellulosimicrobium funkei 27^oC, Enterobacter hormaechei 35^oC and Aspergillus terreus 30^oC. P^H was 7.5 for Cellulosimicrobium funkei, 8 for Enterobacter hormaechei and for 7.5 for Aspergillus terreus. When they were now cultured using the combination of the above parameters Cellulosimicrobium funkei, Enterobacter hormaechei and Aspergillus terreus gave a maximum yield of 7.24mg/l, 7.03mg/l and 6.81mg/l of solubilized potassium respectively. This means that the isolate Cellulosimicrobium funkei yielded more solubilized potassium from feldspar than the reference cultures and could therefore be a better potassium solubilizer.*

Keywords— Aspergillus terreus, Cellulosimicrobium funkei, Enterobacter hormaechei, Potassium, Soil, Solubilizing.

I. INTRODUCTION

Potassium (K) exists in several forms in the soil, including mineral potassium, non-exchangeable potassium and exchangeable potassium and dissolved or solution potassium (K^+ ions). Plants can only directly take up solution potassium (Shanware, 2014).

Soils commonly hold over 20000 ppm of total potassium, plants can use only the exchangeable potassium on the surface of the soil particles and that dissolved in the soil water which often amounts to less than 100 ppm and comprise only 0.1 to 2% of the total potassium (George & Michael, 2002). The rest is held up in insoluble minerals such as feldspar and mica. This is further compounded by the imbalance in fertilizer application where the ratio of potassium to other minerals like phosphorus and nitrogen is very small.

Potassium (K) is a major essential macronutrient for plant growth. The concentrations of soluble potassium in the soil are usually very low and more than 90% of potassium in the soil exists in the form of insoluble rocks and silicate minerals. Potassium (K), one of the seventeen chemical elements required for plant growth and reproduction, is often referred to as "the regulator" since it is involved with over 60 different enzyme systems in plants. Besides its potential to resist drought and disease (Cakmak, 2005; Billore, et al., 2009), it helps in the production of starch, controls root growth and regulates the stomata movement in plant cells and also contributes to quality.

Organic matter after decomposition produces acids like citric acid, formic acid, malic acid, oxalic acid. These organic acids produced, enhance the dissolution of potassium compounds by supplying protons and by complexing Ca^{2+} ions .Previous work has shown organic compounds produced by micro-organisms such as acetate, citrate and oxalate can increase mineral dissolution in soil (Sheng, 2003). Solubilization of potassium occurs by complex formation between organic acids and metal ions such as Fe^{2+}, Al^{3+} and Ca^{2+} (Styriakova, 2003).

In Indian soil, the soluble potassium form is present in approximately 2% and the insoluble form is present in the range of 98% in form of minerals like biotite, feldspar, mica, muscovite and vermiculite (Goldstein, 1994).

This presents an apparent need to search for alternative sources of potassium for plant uptake and use as well as maintaining its availability in the soil for a sustained use. Soil microbes have been reported to play a key role in the natural potassium cycle and therefore, potassium solubilizing microorganisms present in the soil could provide an alternative technology to make potassium available for uptake by plants (Rogers et. al., 1998).

This research was therefore embarked upon to further search out such microorganisms from rhizosphere soil with capabilities of dissolving the insoluble forms of potassium compound to release the soluble potassium for plant use, healthy growth and increased yield.

II. MATERIALS AND METHODS

2.1 Sample Collection

Rhizosphere soil samples from University of Agricultural Science and Lalbagh Garden, both in Bangalore were collected. Samples were collected from six different sites at each location. The collected samples were pooled together to make the composite sample (Parmar and Sindhu, 2013).

2.1.1 Adaptation and Enrichment

The soil samples collected were mixed with insoluble potassium (Feldspar) and incubated for 1 week at room temperature. After adaptation 1 gm of soil was inoculated in 100 ml liquid medium containing 1% glucose, 0.05% yeast extract and 0.5% feldspar and incubated at 37^0 C on 120 rpm for 1 week (Parmar and Sindhu, 2013).

Enriched samples were inoculated after serial dilution up to 10^{-6} on Aleksandrov agar medium constituted as 1% glucose, 0.05% $MgSO_4.7H_2O$, 0.0005% $FeCl_3$, 0.01% $CaCO_3$, 0.2% $CaPO_4$ and 0.5% potassium aluminium silicate, agar 3 % at pH 6.5 (Sugumaran and Janartham, 2007) and incubated at 37^0C for 1 week. Colony exhibiting clear zone of potassium solubilization was selected as potassium solubilizer from the 10^{-5} dilution containing plate. Secondary Screening was carried out on the basis of study of zone of activity of the isolate by using Khandeparkar's selection ratio.

Ratio = D/d = Diameter of zone of clearance / Diameter of growth

2.2 Characterization of Potassium Solubilizing Bacterial Isolate

Bacterial isolate was characterized using different cultural, microscopical and biochemical characteristics (Osman, 2009).

Colony morphology like shape, margin and elevation of the isolate were observed. Gram staining was also done and the slide observed under oil immersion lens (magnification X100) of the microscope.

Standard biochemical tests were then carried out as follows:

2.2.1 Catalase Test

48hrs old bacterial culture was placed on a clean glass slide, drops of 3% H_2O_2 was added and mixed with a tooth pick. Observation of bubble formation indicated the positive test for catalase test (Kumar et al., 2012)

2.2.2 Indole Production

Trypton broth was prepared and inoculated with bacterial culture. This was incubated at 37^oC for 48 hours. 0.5ml of Kovac's reagent was then added to the culture. After 2 minutes observation for appearance of a red colour band at the junction of medium and reagent was made for indole production (Chand et al., 2014).

2.2.3 Methyl red and Voges-Proskauer Test

Three tubes of MRVP broth were taken. Two of the tubes were inoculated with the bacterial culture and one as control (uninoculated). The three tubes were incubated at 35^{o}C for 48 hours. 5 drops of methyl indicator was added into only one of the culture tube. The change in colour was observed for methyl red test (Chand et al., 2014).

To the second culture tube and the control were added 10 drops of VP1 and 2-3 drops of VP2 reagents. The tubes were gently shaken, the cap/plugs were removed and the tubes left for 15-30 minutes to complete the reaction. The colour was then observed for Voges-Proskauer test (Chand et al., 2014).

2.2.4 Starch Hydrolysis

Starch agar plates were prepared and streaked with culture. The culture was allowed to grow at 37^{0}C for 48 hours. Iodine solution was then poured in the culture plate. Colour change around the streaked culture was observed for starch hydrolysis (Chand et al., 2014).

2.2.5 Bacterial Identification- 16 S rRNA Sequencing DNA Extraction

Lysis/homogenization: Cells were grown in monolayer and lysed by suspending 1-3 colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and lysed by repeated pipetting. 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer were added. The content was vortexed and the tubes incubated for 30 minutes at 65˚C in water bath. To minimize shearing the DNA molecules, DNA solutions were mixed by inversion. The tubes were centrifuged for 20 minutes at 14,000 rpm at 10 ˚C. Following centrifugation, the resulting viscous supernatant was transferred into a fresh 2 ml micro centrifuge tube without disturbing the pellet. 600 µl of "B Cube" binding buffer was added to the content and mixed thoroughly by pipetting and the content incubated at room temperature for 5 minutes. 600 µl of the contents was transferred to a spin column placed in 2 ml collection tube. This was centrifuged for 2 minutes at 14,000 rpm and discarded flow-through. The spin column was reassembled and the collection tube then transferred the remaining 600 µl of the lysate. It was then centrifuged for 2 minutes at 14,000 rpm and discarded flow-through. 500 µL "B Cube" washing buffer I was added to the spin column. Centrifuged at 14,000 rpm for 2 minutes and discarded flow-through. The spin column was reassembled and 500 µl "B Cube" washing buffer II added and Centrifuged at 14,000 rpm for 2 mins and discarded flow-through. The spin column was transferred to a sterile 1.5-ml micro centrifuge tube. 100 µl of "B Cube" Elution buffer was added at the middle of spin column. Care was taken to avoid touch with the filter. The tubes were incubated for 5 minutes at room temperature and Centrifuged at 6000 rpm for 1 min. 16. The above mentioned step 14 and 15 were repeated for complete elution. The buffer in the micro centrifuge tube contained the DNA. 17. DNA concentrations were measured by running aliquots on 1% agarose gel. 18. The DNA samples were stored at -20°C until further use.

Reference bacterium *Enterobacter hormaechei* (MTTC Code: 10240) known for solubilizing potassium, were sourced from Microbial Type Collection and Gene Bank (MTTC) of Institute of Microbial Technology, Chandigarh, India. The culture was used as reference culture to compare with the test culture.

The second organism (a fungus) used as reference culture was isolated and identified as *Aspergillus terreus* 28S ribosomal RNA gene, Sequence ID: gb|KF800672.1with accession number as: NCBI is KX775949.

2.3 Solubilization Activity and Optimization Conditions for Efficient K Solubilization

The isolated K solubilizing bacterium and the reference bacteria were tested for their K solubilizing activity under varying conditions of carbon, nitrogen, potassium, temperature and pH sources used.

A loopful of 48 hour old grown bacterial culture was inoculated into 25ml Aleksandrov medium broth in 50ml capacity flask containing either of different sugars: fructose, galactose, glucose and xylose with added flask for control. All the inoculated flasks plus the control were incubated at 28±2^{o}C for 10 days. Same was done for nitrogen sources (beef extract, $NaNO_3$, peptone and urea), potassium sources (KCl and K_2SO_4), varying temperatures (25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C) and varying pH (6.5, 7.0, 7.5and 8.0) (Parmar and Sindhu, 2013).

2.3.1 Quantitative Estimation of Potassium Release

Different concentrations of KCl solution, ranging from 0 – 100 ppm, were used for preparation of standard curve. Sodium cobaltinitrite solution (5ml) was added slowly to each test tube containing varied concentrations of potassium and volume made up to 10ml by adding distilled water. The reaction mixture was incubated at 37^{o}C for 45 minutes to precipitate the potassium and centrifuged at 13,000 rpm for 5 minutes to permit the precipitate to settle down in the tube. The supernatant

was decanted, precipitate collected and washed twice with distilled water and once with absolute ethanol. After washing, 10ml of conc. HCl was added to the precipitate and incubated at 37^{O}C for 15 - 20 minutes to develop the green colour and absorbance was measured at 600nm using the colorimeter.

Following the same procedure and conditions, potassium was estimated in 5ml of culture supernatant, with reference to the standard curve generated. Estimation for each parameter was carried out thrice to obtain the average which was now referenced to the standard curve to obtain the estimate of solubilisation (Rajawat, M. V. S. et al., 2014).

III. RESULTS AND DISCUSSION

3.1 Isolation and Solubilization Activity of Bacteria

A total of 20 different types of colonies were able to grow on Aleksandrov agar. Among these isolated colonies, one bacterial colony was found to make a clearance zone indicating k-solubilization on Aleksandrov agar.

3.2 Morphological and Biochemical Characters of Isolated Strain

The isolate was medium, round, creamy to brownish. It was gram positive rod (fig. 1), starch hydrolysis, indole production, voges-proskauer, methyl red and catalase positive. It was identified to be *Cellulosimicrobium funkei*. The accession number from GenBank is: SUB2919821 Seq1 MF590168. This organism is novel in this regard as it has never been reported for potassium solubilization.

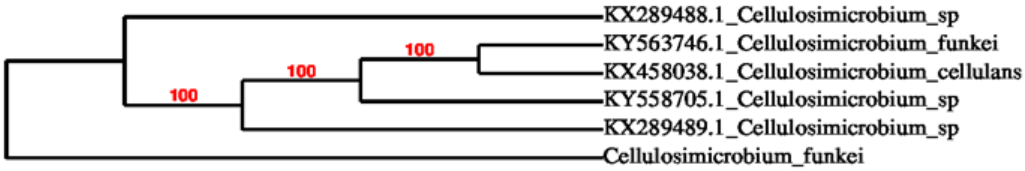
16 S rRNA Sequencing was used

27F 5' AGAGTTTGATCMTGGCTCAG 3'

1492R 5' AGAGTTTGATCMTGGCTCAG 3'

Organism: *Cellulosimicrobium funkei*

Gene Sequence of *Cellulosimicrobium funkei*



LAYOUT 1: Phylogenic tree of *Cellulosimicrobium funkei*

TABLE 1
K SOLUBILIZATION ZONE FORMATION BY ISOLATE *CELLULOSIMICROBIUM FUNKEI*

Isolate	Diameter of Zone of clearance (D) in mm	Diameter of growth of Colony (d) in mm	D/d Ratio
Cellulosimicrobium funkei	12	07	1.71

Khandeparkar's ratio: D/d. D = Diameter of zone of clearance, d = Diameter of growth of isolate

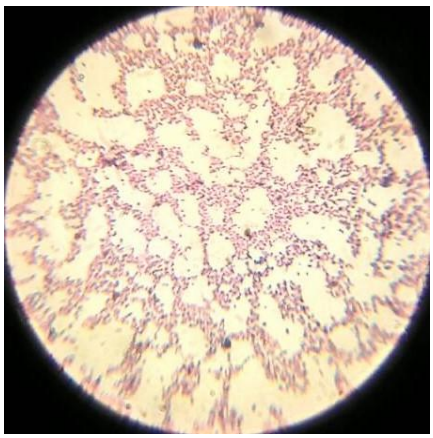


FIGURE 1: Microscopic View of *Cellulosimicrobium funkei* (2A) (Magnification X100)

3.3 Potassium (K) Solubilization Activity

The isolate (*Cellulosimicrobium funkei*) solubilized potassium better than the reference organisms in the four carbon sources of fructose, galactose, glucose and xylose. It solubilized best with glucose (7.04mg/l) while the reference organisms were 7.15mg/l and 6.91mg/l for *Enterobacter hormaechei* and *Aspergillus terreus* respectively as seen in Table 2 and fig. 2. This compares well with the work of Parma and Sindhu (2013) that potassium solubilization by two bacterial isolates was more in glucose supplemented medium than galactose, xylose or arabinose. Etesami, H. et al (2017) also found that the best carbon source for solubilisation of potassium was found to be glucose.

TABLE 2
SOLUBILIZATION BY CULTURES IN GLUCOSE FORTIFIED MEDIUM

Amount of Glucose Added (g)	K – Solubilized (mg/l)		
	***Cellulosimicrobium funkei* (2A)**	***Enterobacter hormaechei* (RF)**	***Aspergillus terreus* (2B)**
Control	0.12	0.42	0.22
1	0.23	1.31	2.02
2	2.51	3.21	2.10
3	2.40	3.60	3.81
4	3.15	4.16	3.60
5	4.22	5.47	4.51
6	5.30	6.89	6.28
7	7.04	7.15	6.91
8	6.91	6.25	6.80
9	6.52	6.27	6.32
10	4.66	5.78	4.31

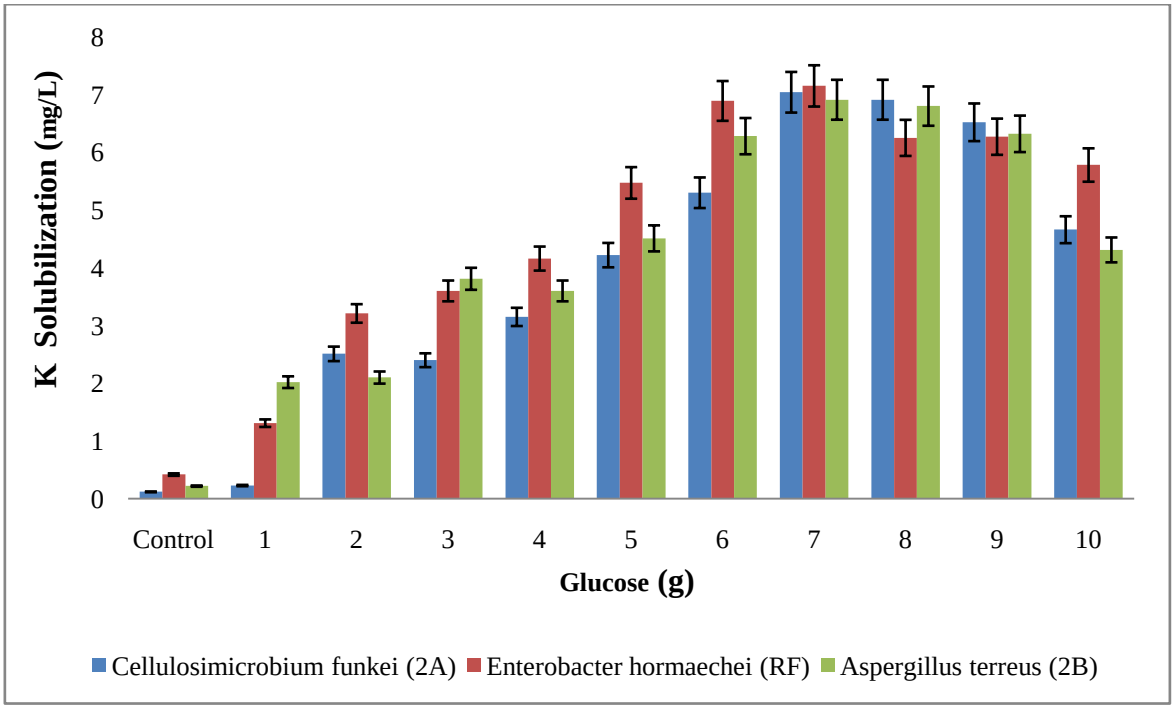


FIGURE 2: Graph showing Solubilization by cultures in glucose fortified medium

Solubilization by the *Cellulosimicrobium funkei* was best in urea culture as nitrogen source (5.45mg/l) and favourably compared with the reference organisms: *Enterobacter hormaechei* 5.38mg/l and *Aspergillus terreus* 6.33mg/l in the entire nitrogen sources as presented in table 3 and fig 3.

TABLE 3
SOLUBILIZATION BY CULTURES IN UREA FORTIFIED MEDIUM

Amount of Urea Added (g)	K – Solubilized (mg/l)		
	Cellulosimicrobium funkei (2A)	*Enterobacter hormaechei* (RF)	*Aspergillus terreus* (2B)
Control	0.86	1.01	0.02
1	2.22	2.41	1.57
2	3.21	2.56	2.71
3	4.92	3.87	2.77
4	5.00	4.85	3.94
5	5.21	4.93	5.03
6	5.45	5.38	5.26
7	5.41	4.92	6.33
8	4.82	4.83	2.78
9	3.80	4.51	2.50
10	3.52	2.60	2.13

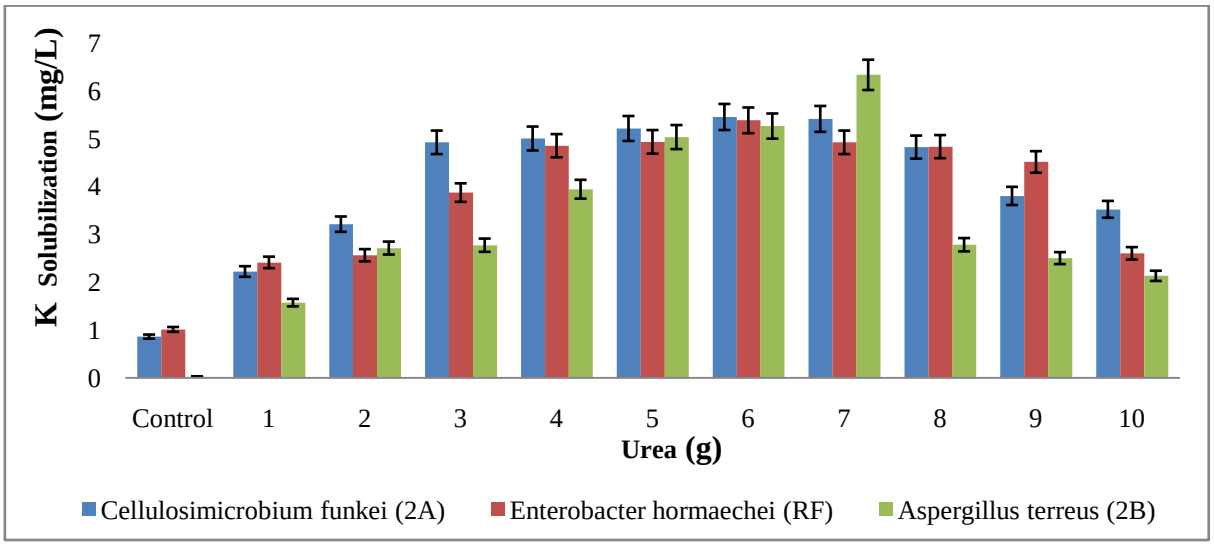


FIGURE 3: Graph showing Solubilization by cultures in Urea fortified medium

The three organisms performed best with the potassium chloride (KCl) medium as potassium source but *Cellulosimicrobium funkei* surpassed the reference organisms with solubilization of 10.23mg/l as against 8.05mg/l for *Enterobacter hormaechei* and 9.11mg/l for *Aspergillus terreus* respectively as shown in Table 4 and fig. 4.

TABLE 4
SOLUBILIZATION BY CULTURES IN KCL FORTIFIED MEDIUM

Amount of KCl Added (g)	K – Solubilized (mg/l)		
	Cellulosimicrobium funkei (2A)	*Enterobacter hormaechei* (RF)	*Aspergillus terreus* (2B)
Control	0.04	1.22	1.10
1	1.81	2.07	2.51
2	2.66	3.15	3.88
3	5.24	4.22	5.25
4	5.41	5.31	6.11
5	6.08	5.89	8.01
6	8.11	6.53	8.11
7	8.62	6.73	8.13
8	10.23	8.05	9.11
9	9.31	6.28	8.37
10	7.22	5.34	7.89

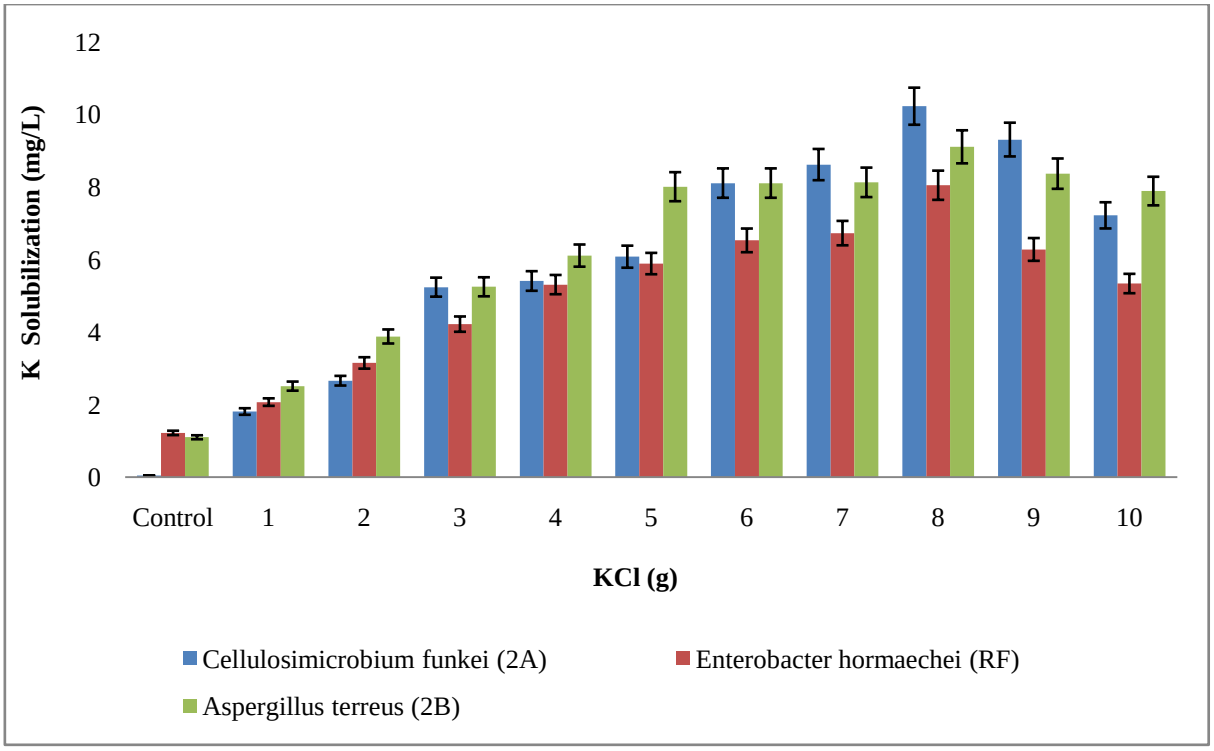


FIGURE 4: Graph showing Solubilization by cultures in KCl fortified medium

It was noticed that the organisms gave best yield at different temperatures. 27°C, 35°C and 30°C for *Cellulosimicrobium funkei, Enterobacter hormaechei* and *Aspergillus terreus* respectively as shown in fig. 5. The temperature (27°C) at which the *Cellulosimicrobium funkei* solubilized best compares favourably with the finding of Prajapati and Modi (2012) which showed *Bacillus* solubilizing insoluble potassium well in aleksandrov medium at a temperature range of 25°C to 35°C. Etesami, H. et al (2017) also reported a better solubilization of potassium in glucose as carbon source at 35°C.

Cellulosimicrobium funkei solubilized best at pH range of 7.5, *Enterobacter hormaechei* at 8 and *Aspergillus terreus* at 7.5 fig. 6. Again, the performance of the isolate (*Cellulosimicrobium funkei*) at pH 7.5 is same with the finding of Prajapati and Modi (2012) with *Bacillus spp*.

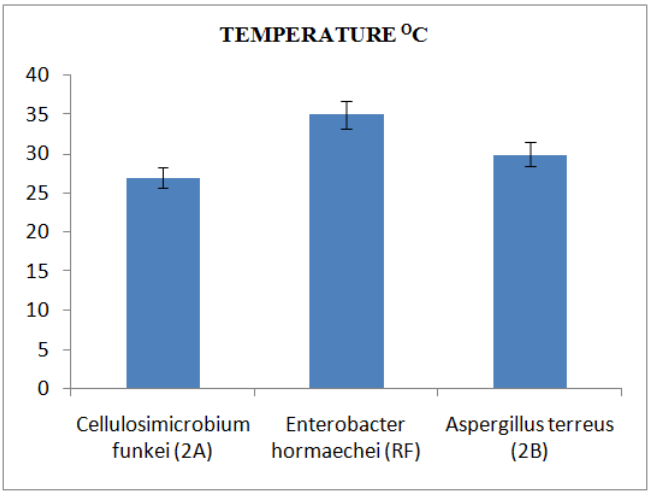


FIGURE 5: Graph showing Solubilization by cultures at their respective temperatures

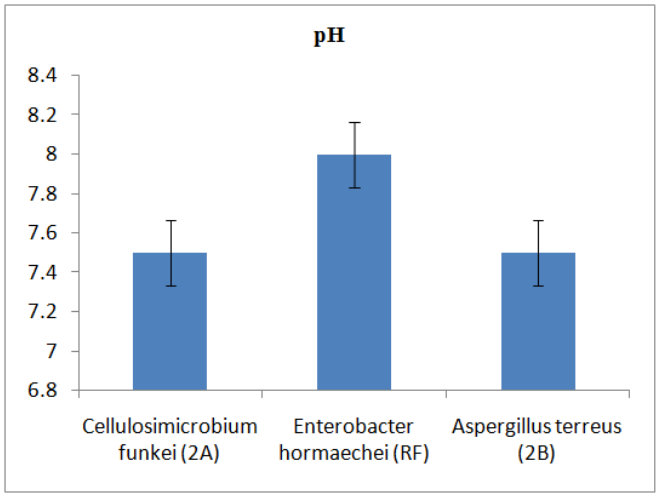


FIGURE 6: Graph showing Solubilization by cultures at their respective pH

When the three microorganisms were cultured under a combined condition of all parameters at the values earlier shown they yielded solubilized potassium at the respective amount of 7.24mg/l, 7.03mg/l and 6.81mg/l by *Cellulosimicrobium funkei*, *Enterobacter hormaechei*, and *Aspergillus terreus*. This is shown on Table 5.

TABLE 5
SUMMARY OF SOLUBILIZATION BY THE CULTURES

Culture	Glucose (g)	UREA (g)	KCl (g)	Temperature ^{O}C	P^{H}	K – Solubilized (mg/l)
Cellulosimicrobium funkei (2A)	8	6	8	27	7.5	7.24
Enterobacter hormaechei (RF)	8	6	8	35	8	7.03
Aspergillus terreus (2B)	8	7	8	30	7.5	6.81

IV. CONCLUSION

In this study, an isolate showed zone of potassium solublization in aleksandrov medium using feldspar as the insoluble potassium source. Morphological and biochemical tests on the isolate from the rhizosphere soil that showed solubilization activity pointed to its identity as *Cellulosimicrobium funkei* a novel organism in potassium solubilization. Optimization tests with varying concentrations of carbon, nitrogen, potassium sources, temperature and pH showed encouraging solubilization ability by the isolate and it compared favourably and in some instances better than the reference organisms.

This agrees with some other researches carried out like Parmar and Sindhu (2013) who reported some bacterial species like *Enterobacter hormaechei, Paenibacillus glucanolyticus , Bacillus edaphicus* and *Bacillus circulans* as potassium solubilizers. Just as Etesami, H. etal (2017) also reported that KSB are usually present in all soils but that their number, diversity and ability for K solubilisation vary depending on upon the soil and climatic conditions. They mentioned some bacteria like *Paenibacillus spp*. and *Bacillus circulans* as having capacity to solubilize potassium.

Therefore, more studies on *Cellulosimicrobium funkei* is needful and worthwhile to further ascertain its solubization capabilities on potassium compounds and others. This could add to the number of candidates for the production of biofertilizers to enrich our potassium starved soils for the better health, growth and production of plants. This will consequently add to more abundance of healthy food for the growing human populace.

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