Biocatalysis and Agricultural Biotechnology 20 (2019) 101201

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

The bacterial biodegradation of soil lecithin into biofertilizer catalyzed by plant micro nutrients- molybdenum, manganese, and zinc ions

Sangita Saha[a], Niranjan Kumar Mandal[b], Tanusri Mandal[a,*]

[a] Dept. of Biotechnology, Vidyasagar University, Midnapore, West Midnapore, 721102, West Bengal, India
[b] Department of Electrical Engineering, University of Engineering & Management, New Town, Kolkata, 7000160, India

ARTICLE INFO

Keywords:
Lecithin
Molybdenum
Zinc
Manganese
Biodegradation
Biofertilizer

ABSTRACT

The main object of this paper is to carry out experimental study to find the biodegradation of an insoluble phospholipid, lecithin by bacterial strains into water soluble phosphate compounds and other valuable products that are beneficial for biotic and abiotic systems of the soil. Lecithin is present in soil and phosphorus of phosphate group of lecithin cannot be consumed by the plants. To meet this objective three different bacterial strains, like- SMS33, SKPS32, and SKPS41 have been isolated and identified as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Pseudomonas* sp. respectively by 16S rRNA sequencing and nucleotide BLAST analysis. In this investigation, three different plant micro-nutrients like molybdenum, zinc, and manganese ions have been used separately as bio-activator for lecithin bio-degradation with the ease of enzyme secretion. The degraded by-products have also been identified by LC-MS analysis. It has been observed that SMS33 bacteria in presence of ammonium molybdate catalyst shows complete bio-degradation of lecithin molecule. The degraded products of lecithin has been tested with *Abelmoschus esculentus* seeds to check their germination percentage, vigor index value, chlorophyll concentration, fruit yields etc. In comparison, molybdenum ion is the highest activating agent for the secretion of enzymes, lecithin degradation, and plant growth regulator. The results have been tabulated, shown graphically and discussed.

1. Introduction

Soil is the reservoir of several macro and micro nutrients, which are valuable for plants, microorganisms, and different animal creatures for their growth and development. Majority of nutrients remain in their respective forms of complex structure in the soil. Plants cannot assimilate these macro and micro nutrients. But there are several soil microorganisms which can convert complex form into simple form for bioavailability of the nutrients. Phosphorus is one of the most important macronutrient for growth of plants. It has been reported that soil contains 1% of lecithin with respect to the total soil phosphorus (Szember, 1960; Tarafdar and Claassen, 1988). Lecithin is a compound containing water insoluble phosphorus. So, instead of synthetic phosphate fertilizer which is applied by the farmers, plants can consume phosphorus from this natural source, i.e. soil. Plant root's enzymatic activities or soil microorganisms convert lecithin into bioavailable form through biodegradation process. For the growth and developments, plants also need other micronutrients such as molybdenum, zinc, and manganese salts. This novel work aims to design a kind of bioinoculum which will (i) degrade lecithin (ii) increase the phospholipase enzymatic activity by molybdenum, zinc and manganese ions separately, (3) increase the lecithin degrading ability by molybdenum, zinc and manganese ions separately into soluble phosphorus compounds and other valuable products. In this study, the bacterial strains such as *Bacillus subtilis*, *Pseudomonas aureginosa*, and *Pseudomonas* sp. have been selected for the bacterial degradation of lecithin and its utilization for the seed germination and fruits production.

2. Materials and methods

Three bacterial strains, which have been used for the experimental study, are SMS33, SKPS32, and SKPS41. The NCBI (https://www.ncbi.nlm.nih.gov/) accession numbers of SMS33, SKPS32, and SKPS41 are KX352265, KX352266 and KX352267 respectively. Instead of lecithin, soy-lecithin has been used for this experiment. Soy-lecithin and all other laboratory chemicals have been purchased from HIMEDIA, Mumbai Pvt. Ltd., India.

* Corresponding author.
E-mail addresses: sangitasaha0805@gmail.com (S. Saha), niranjan.mandal@uem.edu.in (N.K. Mandal), tanusrimandaloist@gmail.com (T. Mandal).

https://doi.org/10.1016/j.bcab.2019.101201
Received 16 February 2019; Received in revised form 16 May 2019; Accepted 12 June 2019
Available online 13 June 2019
1878-8181/

2.1. Formation of phylogenetic tree

Topmost five homologs for each of the three bacterial strains from nucleotide BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) analysis have been considered for sequence alignment, and phylogenetic tree generation in MEGA 6.0 software.

2.2. Biodegradation of lecithin on agar plate

In this study, biodegradation activity on agar plate have been conducted in four different processes. First one: The study have been conducted for agar plate containing soy-lecithin in place of calcium-triphosphate and other constituents of standard Pikovskaya media (Pikovskaya, 1948). Second one: The study have been conducted for agar plate containing soy-lecithin in place of calcium-tri-phosphate, other constituents of standard Pikovskaya media, and 401 ppm of molybdenum ion in ammonium molybdate. Third one: The study have been conducted for agar plate containing soy-lecithin in place of calcium-tri-phosphate, other constituents of standard Pikovskaya media, and 9 ppm of zinc ion in zinc nitrate. Fourth one: The study have been conducted for agar plate containing soy-lecithin in place of calcium-triphosphate, other constituents of standard Pikovskaya media, and 25 ppm of manganese ion in manganese sulphate. At first, the CFU/ml has been counted for the first sample by using the formula [CFU/ml = (number of colonies X total dilution factor)/volume of culture plate] (https://orbitbiotech.com/how-to-calculate-cfu-colony-forming-unit-cfu-colony-forming-unit-cfu-ml-cfu-g-cell-count-microbial-counting/) and that is 10^4 CFU/ml. Afterwards, by varying the dilution factor and volume of bacterial culture on agar plate the CFU/ml has been adjusted to 10^4 CFU/ml for each of the second, third and fourth sample. Each of the three 50 µl of previously mentioned bacterial culture (10^4 CFU/ml) has been spot plated separately on sterilized agar plate containing media composition of previously mentioned under first, second, third, and fourth part under this section. All the plates have been inspected; data value recorded and solubilization efficiency (Nguyen et al., 1992; Seshadri et al., 2002) have been counted on 20^{th} day with respect to the day of inoculation by using the following formula:

Solubilization Efficiency = (solubilization diameter/ growth diameter) X 100

2.3. Effect of temperature on bacterial growth and lecithin biodegradation study

In this study, the first two media compositions, except agar mentioned under “biodegradation of lecithin on agar plate” have been used. Different temperatures like 20 °C, 25 °C, 30 °C, and 35 °C have been considered for this study. Each of the three 50 µl of previously mentioned bacterial culture (10^4 CFU/ml) has been inoculated separately, except control. Bacterial growth has been encountered followed by counting the soluble phosphate in broth with respect to the control according to Murphy et al. (Murphy and Riley, 1962).

2.4. Dynamic variation of bacterial activity

In this activity test, the four media compositions, except agar mentioned under “biodegradation of lecithin on agar plate” have been used. Each of the three 50 µl of previously mentioned bacterial culture (10^4 CFU/ml) has been inoculated separately, except control. Different parameters like pH variation, bacterial growth, soluble phosphate count, and phospholipase activity have been considered for this study. Each of 2^{nd} to 18^{th} day alternatively bioinoculum, and control samples have been collected. The bacterial growth has been measured followed by centrifugation at 10000 rpm for 10 min. The supernatant has been used for the measurement of pH, soluble phosphate concentration, and phospholipase activity of the bioinoculum and control separately. Each and every data have been recorded from each of the 2^{nd} to 18^{th} day alternatively. Murphy et al. (Murphy and Riley, 1962) and Kuroshima et al. (Kuroshima and Hayan, 1982) have been followed for this purpose.

2.5. Liquid chromatography-mass spectrometry (LC-MS) analysis

Cell free extract and control have been used for this study. Mane et al. (2013) has been followed for LC-MS analysis. In this investigation, Intersil C18 column has been used for this LC-MS analysis. The proportion of water, acetonitrile and methanol is used in the ratio of (10: 60: 30) and it has been taken as mobile phase. The flow rate has been kept at 1 ml/min at 210 nm.

2.6. Biofertilization activity test

In this test, PVK + L, PVK + L + AMo, PVK + L + ZN, PVK + L + SMS33, PVK + L + SKPS32, PVK + L + AMo + SMS33, PVK + L + ZN + SMS33, PVK + L + AMo + SKPS32, and PVK + L + ZN + SKPS32 have been used separately as bioinoculum for non-sterilized soil treatment. Selection of bioinoculum have been conducted on the basis of aforesaid experimental results. Ten pots of non-sterilized soil with marked serial number have been taken separately for conducting the experiment. Soil has been inoculated at room-temperature with non-sterilized water, three different composition of 10^{th} day inoculum of PVK + L, PVK + L + AMo, PVK + L + ZN, six different composition of 10^{th} day bioinoculum of PVK + L + SMS33, PVK + L + SKPS32, PVK + L + AMo + SMS33, PVK + L + ZN + SMS33, PVK + L + AMo + SKPS32, and PVK + L + ZN + SKPS32 separately for one week. In this case, control has been selected as non-sterilized water treated soil. Here, no positive or negative control has been used for this experimental study. Non-sterilized *Abelmoschus esculentus* seeds have been placed 1 cm beneath from the upper layer of treated soil in each pot separately. During this time soil has been kept little bit wet by spraying non-sterilized water only. Spraying of non-sterilized water has been maintained throughout the whole experimental tenure. After a week seed germination has been recorded. On 30^{th} day data has been recorded from each set. Ashkan et al. and Mehta et al. (Abbasian and Moemeni, 2013; Mehta et al., 2015) have been followed for calculating the germination percentage and seedling vigor-index. The whole experiment has been conducted in the pots. Bambara Sylvie Karumeyi's thesis (Karumeyi, 2009.) has been followed for calculation of total chlorophyll in dried plant material, collected from each set by using following formula:

Total chlorophyll content = $(20.2 \times D_{645} + 8.02 \times D_{663})$

Here, D_{645} and D_{663} are absorbance at 645 nm and 663 nm respectively.

2.7. Statistical analysis

Standard deviation has been calculated by using the Microsoft Office Excel 13.0. On the other hand, IBM SPSS 20.0 package has been used for statistical analysis. One way analysis of variance (ANOVA) has been used to study the significance of the outcome. Here, LSD and Duncan method’ under the post-hoc test method at $p < 0.5$ significance level has been used.

3. Results

Average value of three times consecutive results have been finally considered.

Fig. 1. Maximum likelihood algorithm with bootstrap value has been considered for tree construction.

3.1. Phylogenetic tree

"T92 + G" with lowest BIC value has been found as best fit model for the following phylogenetic tree. Among the three bacterial strains, it has been found that SMS33 is totally diverged out from the main branch. Other two strains remains with its nearest homologs. The graphical representation of them has been shown in the Fig. 1.

3.2. Agar plate inspection record

Fig. 2 Shows the metallic effect on bacteria for their solubilization efficiency of water insoluble soy-lecithin, organophosphate compound separately. Among three bacterial strains, SMS33 and SKPS32 show the effect in the order of molybdenum > zinc > manganese, whereas SKPS41 does not differ on solubilization efficiency for any of the metal presence. However, definitely, all of the bacterial strains show significant difference on solubilization efficiency with respect to any of the absence of heavy metals.

3.3. Bacterial growth and lecithin biodegradation study at different temperatures

Both the temperatures, 20 °C and 25 °C haven't given any significant effect on bacterial growth as well as lecithin degradation for each of three bacterial strains used in this experiment for each of both types of media composition like PVL + L and PVL + L + AMo. On the other

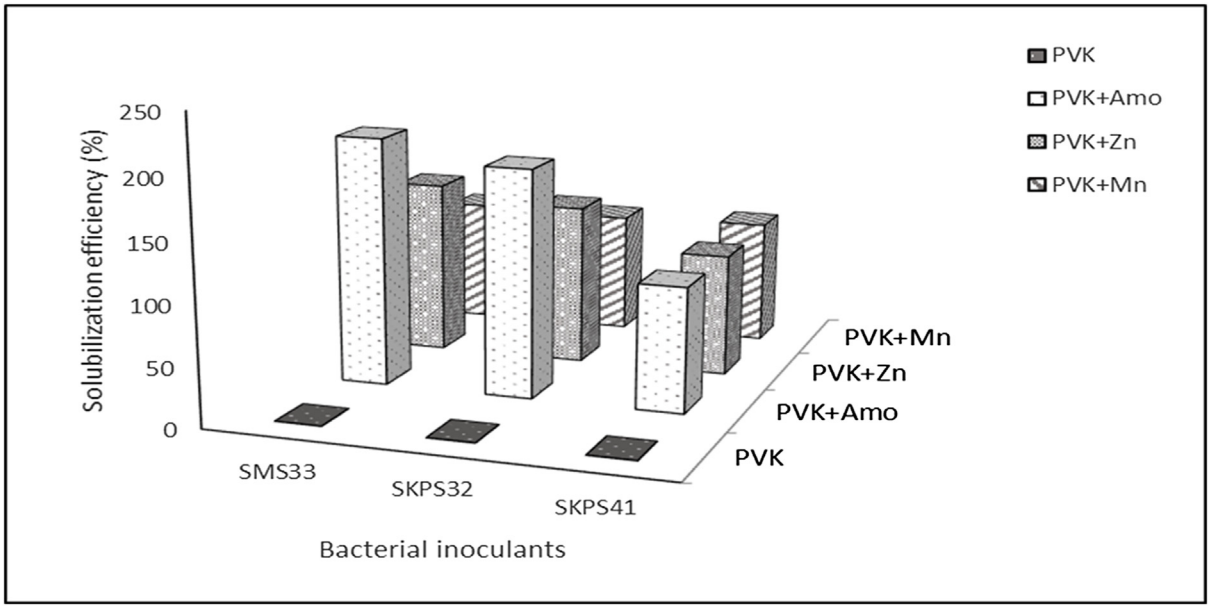

Fig. 2. Effect of solubilization efficiency of the water insoluble soy-lecithin by bacterial isolates on agar plate.

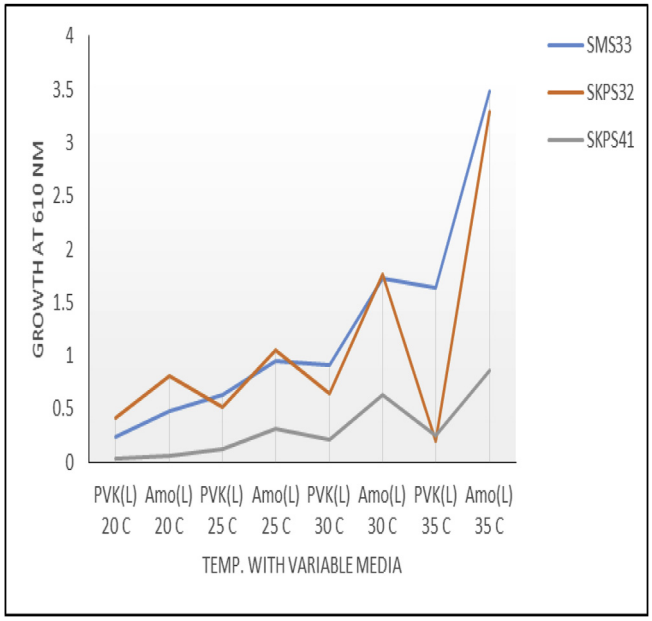

Fig. 3a. The variation of bacterial growth at different temperatures in presence of different bio inoculants.

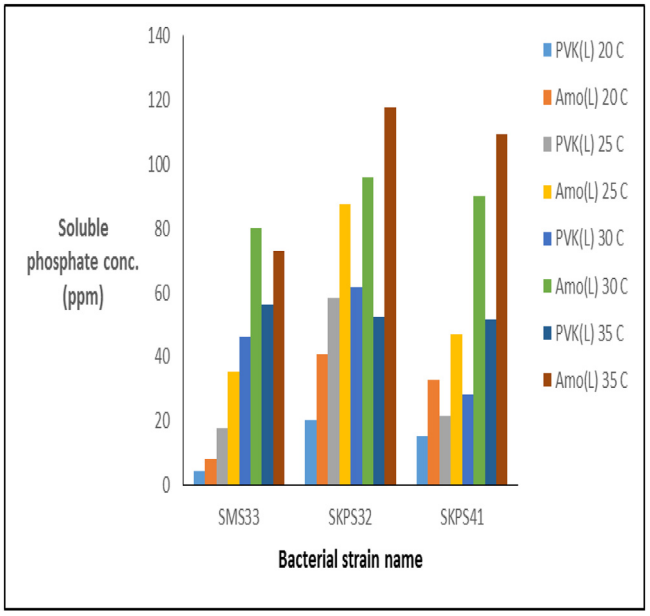

Fig. 3b. The variation of soluble phosphate conc. (ppm) at different temperatures in presence of different bio inoculants.

side, 35 °C has been found as optimal bioactive temperature for both SMS33 and SKPS41strains whereas 30 °C has been found as optimal bioactive temperature for SKPS32 bacterial strain. Informed data analysis on Fig. 3a. and Fig. 3b. for bacterial biomass variation and soluble phosphate concentration in broth solution respectively.

3.4. Dynamic variation of bacterial activity

Fig. 4a. Shows the pH variation for eighteen days. Drops in pH has been recorded for each of the bioinoculum tested with respect to each set of control used in this case. Fig. 4b. Shows the bacterial biomass variation for eighteen days. Molybdenum ion helps for better growth of SMS33 strain with respect to other set of bioinoculum. Fig. 4c. Shows the correlation study on soluble phosphate concentration in ppm with enzyme activity of different composition bioinoculum. The presence of soluble phosphate in broth is the result of degradation soy-lecithin by bacterial strains. From these figures, it has been found that SMS33 in presence of molybdenum ion performed best with respect to the other tested set of bioinoculum composition.

3.5. Liquid chromatography-mass spectrometry (LC-MS) analysis

Different TIC (Total ionization chromatogram) of LC-MS analysis result of cell-free extract along with the control have been shown in the Fig. 5a., Fig. 5b., Fig. 5c., and Fig. 5d.

3.6. Biofertilization activity test

Table 1 and Table 2 give the details of pot experiment with standard deviation values for each of the significant results. No significant result has been found from PVK + L, PVK + L + AMo, PVK + L + ZN, PVK + L + SMS33, PVK + L + SKPS32, and PVK + L + ZN + SKPS32. Hence, their results have not been included in the above mentioned table. It has been found that the bioinoculum PVK + L + AMo + SMS33, and PVK + L + ZN + SMS33 give better result with respect to other bioinoculum used and control too. The F values for seedling vigor index, total chlorophyll content and germination percentage for SMS33 strain are 159667, 1204, and 80.56 respectively at $p < 0.5$ and df (degree of freedom) for treatment is equal to 3.

4. Discussion

4.1. Phylogenetic analysis

Phylogenetic tree clearly reveals that SKPS32 and SKPS41 strains are very much symmetrical to BLAST result i.e. their properties reflect very much similar activities of *Pseudomonas* sp. whereas SMS33 reflects its divergent nature with respect to the *Bacillus subtilis*. It indicates that the strain is somewhat different from *Bacillus subtilis*.

4.2. Agar plate inspection analysis

In this case, all the cations like molybdenum, zinc, and manganese sensitize the newly isolated bacterial cultures for better biodegradation of soy-lecithin molecule. Effects of molybdenum on bacterial culture sensitization except SKPS41 is more than zinc ion followed by manganese ion. However, for SKPS41 the effect of molybdenum, zinc, and manganese ion is almost same. It implies that SMS33 and SKPS32 may follow different kind of degradation pathway for different types of cation presence, i.e., molybdenum, zinc, and manganese ions whereas SKPS41 strain follow same kind of degradation pathway for different types of cationic presence. Strictly we have not studied the mechanism pathway of lecithin degradation. Alternatively, their sensitization effect on bacterial culture is different in some extent.

4.3. Analysis of bacterial growth and lecithin biodegradation study at different temperatures

All of the used bacterial cultures grow well along with better biodegradation with the increase of temperature i.e. in this case, maximum temperature is 35 °C. In this case also, presence of molybdenum ion show better growth along with better biodegradation with respect to its absence in broth cultures. Here, we have observed from our experiment that presence of molybdenum at higher temperature (35 °C) accelerate biodegradation of lecithin. This implies that the newly isolated strain can actively survive and biodegrade lecithin in the tropical and subtropical region of the world.

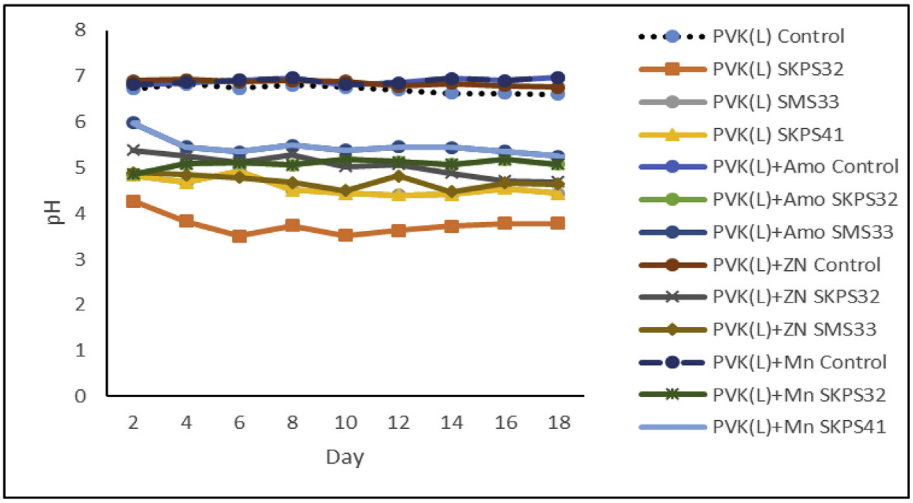

Fig. 4a. Comparative study on pH variation throughout the 18 days in presence of salt of molybdenum, zinc, and manganese separately with respect to their absence.

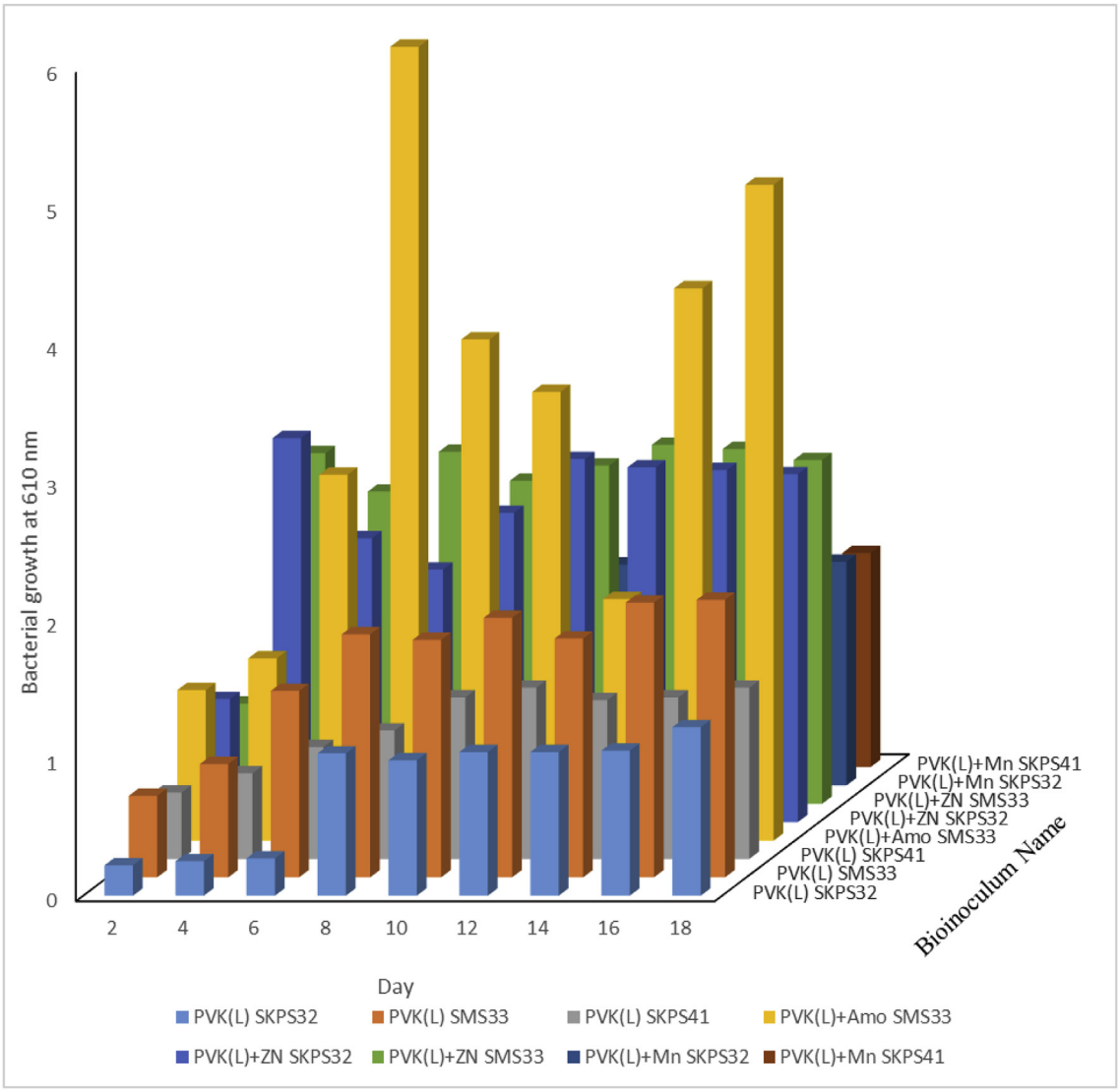

Fig. 4b. Comparative study on bacterial growth throughout the 18 days in presence of salt of molybdenum, zinc, and manganese separately with respect to their absence.

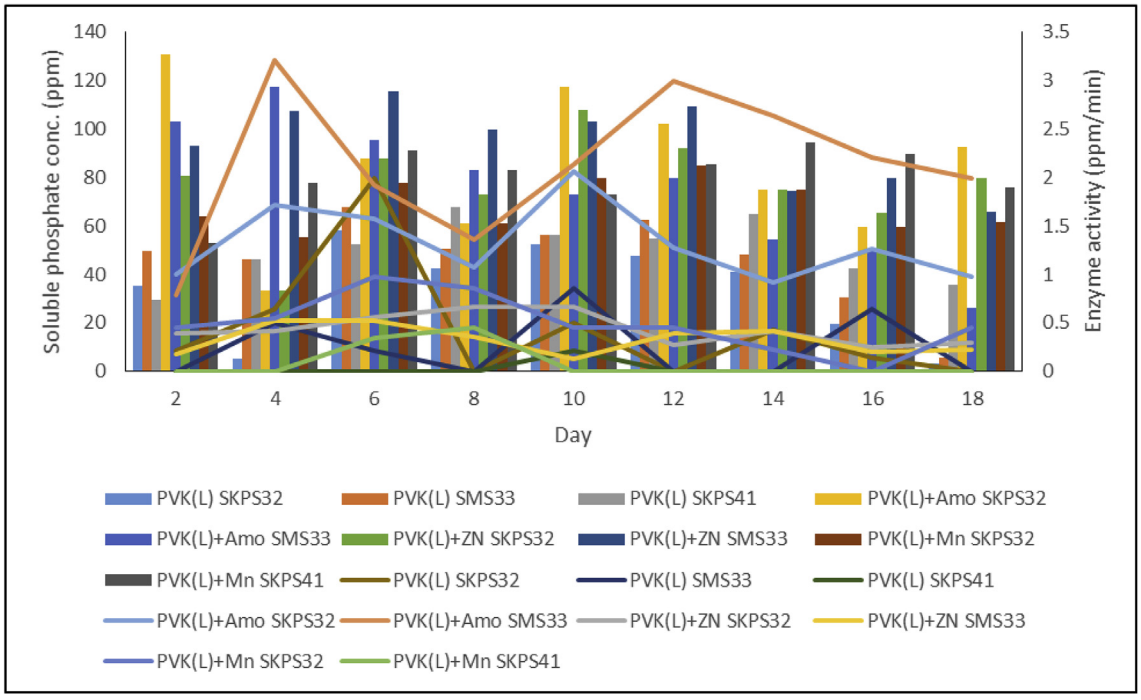

Fig. 4c. Comparative study on soluble phosphate conc. (ppm) with the effect of phospholipase enzyme activity throughout the 18 days in presence of salt of molybdenum, zinc, and manganese separately with respect to their absence.

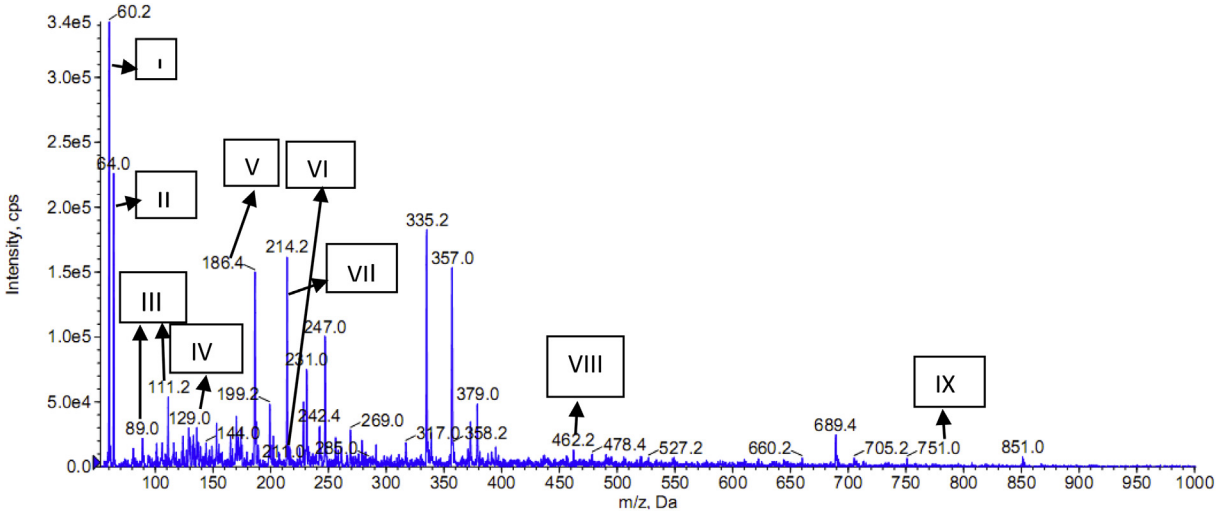

Fig. 5a. LC-MS spectrum of SMS33 culture supernatant containing PVK (L). The TIC contains peaks of (I) Acetic acid [M], (II) Ethanol $[M + NH_3 + H]^+$, (III) Butyric acid $[M+H]^+$, and [M+Na], (IV) choline $[M + Na + 2H]^+$, (V) Diglyceride $[M + K + H]^+$, (VI) L-α-glycerophosphate [M+K], (VII) Gluconic acid $[M + NH_3 + H]^+$, (VIII) Lysolecithin $[M + Na + H]^+$, and (IX) Phosphotidyl ethanolamine $[M + NH_3]$.

4.4. Analysis on dynamic variation of bacterial activity

The drop in pH in bacterial culture indicates the production of acids with respect to control. Bioinoculum with respect to control reveals that bacterial cultures produce organic or inorganic acids.

After ten days, the growth of bacterial cultures deviates implies that use of 10^{th} day bioinoculum will be more effective than its older or younger culture.

The presence of molybdenum cation catalyzes the phospholipase activity, which further help soy-lecithin degradation ability of bacterial strains. Thus, such mechanism, increases the nutrient availability, i.e. phosphorus to bacterial strains. In this case, SMS33 strain shows better result followed by SKPS32 strain. In addition, the presence of zinc and manganese ion catalyzes the enzyme activity to some extent and which with no doubt help in soy-lecithin biodegradation. This is the reason why we get the difference in agar plate results in presence of different ions. This is also the reason why we do not have any positive result in bioinoculum containing no molybdenum, zinc, and manganese ions.

4.5. Analysis on liquid chromatography-mass spectrometry (LC-MS)

It has seen from the LC-MS analysis that the presence of acetic acid, butyric acid, gluconic acid, molybdic acid, monomolybdophosphoric acid as by-products implies that decrease in pH value reflects the production of such kinds of acids. The bacterial strain SMS33 completely degrades lecithin into different products like lysolecithin, choline, L-α-glycerophosphate etc. In presence of ammonium molybdate this same strain produces phosphorus containing products like mono-

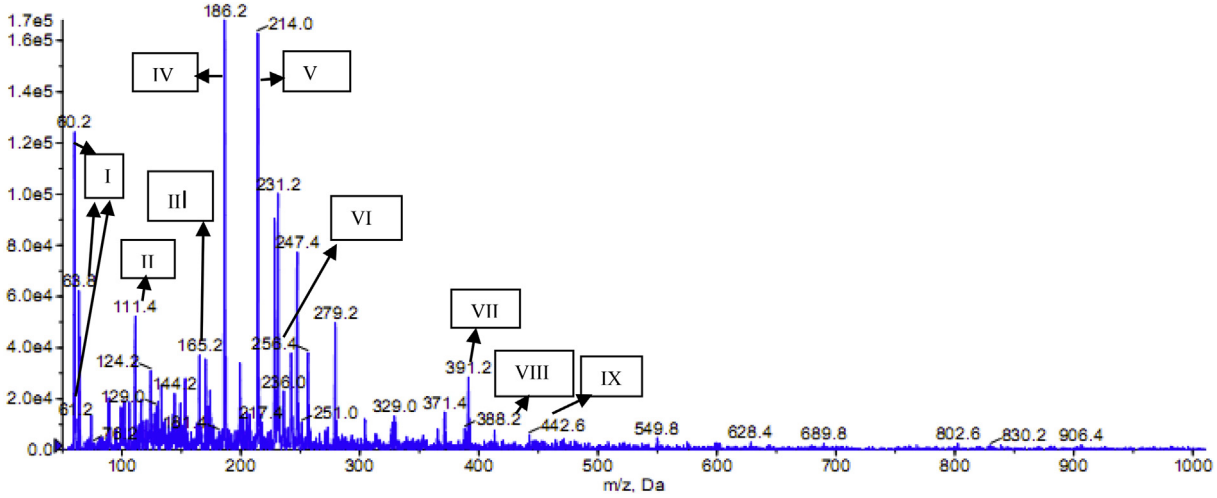

Fig. 5b. LC-MS spectrum of SMS33 culture supernatant containing PVK (L) + AMo. The TIC contains peaks of (I) Acetic acid [M], $[M+H]^+$ and $[M+4H]^+$, (II) Butyric acid [M+Na], (III) Molybdic acid $[M+3H]^+$, (IV) Diglyceride $[M + K + H]^+$, (V) Gluconic acid $[M + NH_3+H]^+$, (VI) Monomolybdophosphoric acid $[M+H]^+$, (VII) Phosphotidyl inositol $[M+2H]^+$, (VIII) Phosphotidyl serine $[M+3H]^+$, and (IX) Lysolecithin $[M+2H]^+$.

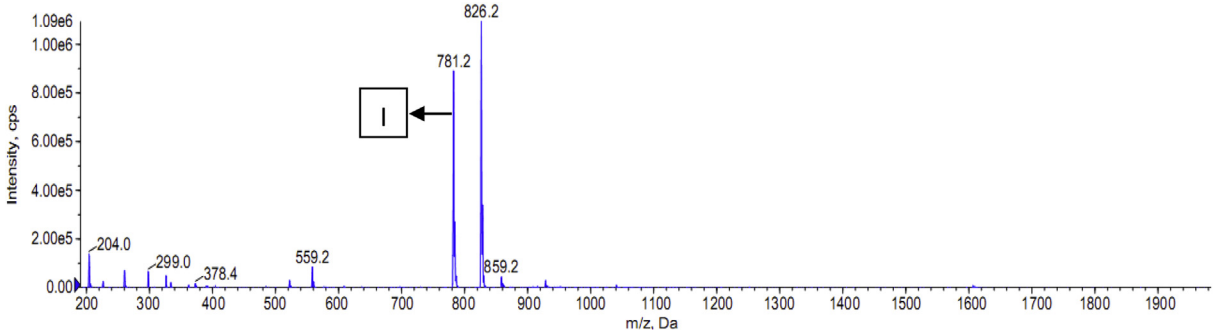

Fig. 5c. TIC of LC-MS analysis of same non-culture supernatant (control) of PVK (L) contains the peaks of (I) Lecithin [M+Na].

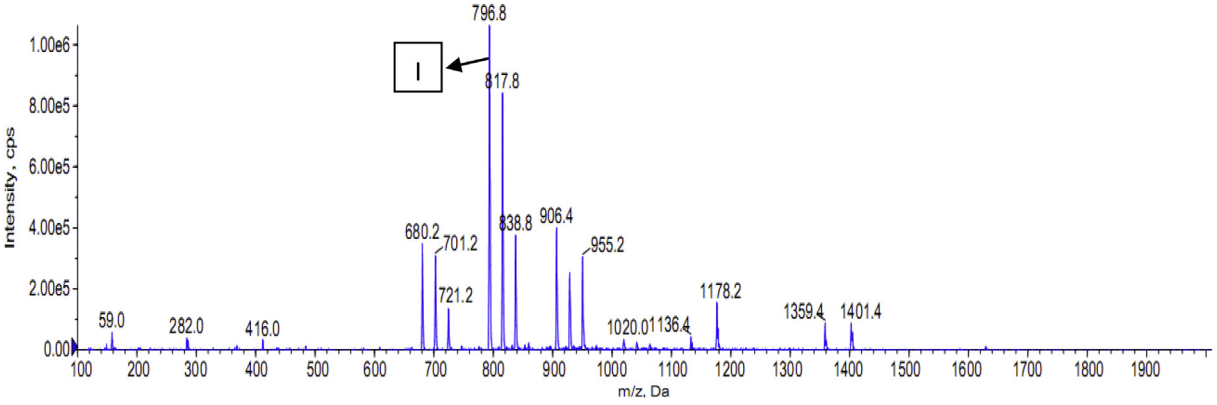

Fig. 5d. TIC of LC-MS analysis of same non-culture supernatant (control) of PVK (L) +AMo contains the peaks of (I) Lecithin [M+K].

Table 1
Yield details of pot experiment.

Bioinoculum name	No of fruit yield	Average fruit weight (gms)	Day with respect to first yield
PVK + L + AMo + SMS33	4	27	52
PVK + L + ZN + SMS33	3	25	50
PVK + L + AMo + SKPS32	0	0	0
Control	3	20	75

molybdophosphoric acid, phosphotidyl inositol, phosphotidyl serine etc. But in absence of molybdenum ion strain can produce phosphorus containing compounds except L-α-glycerophosphate and phosphotidyl ethanolamine. Thus, data also supports the higher concentration of phosphorus in liquid broth containing both bacteria and molybdenum salts than without metal salt. No significant peaks in LC-MS data have been found in absence of both metal and bacteria or in presence of metal ion only.

4.6. Analysis on biofertilization activity

The bacterial Strain SMS33 along with ammonium molybdate and

Table 2
Parameter of biofertilization activity details of pot experiment.

Bioinoculum name	Germination Percentage	Seedling Vigor Index	Total chlorophyll concentration (ppm)
PVK + L + AMo + SMS33	72.22 ± 9.62	1480.89 ± 3.41	15.39 ± 0.50
PVK + L + ZN + SMS33	33.33 ± 5	1373.33 ± 3.73	13.28 ± 0.52
PVK + L + AMo + SKPS32	16.67 ± 5	741.67 ± 3.62	34.9 ± 0.55
Control	16.67 ± 5	433.33 ± 3.70	4.19 ± 0.15

zinc ions show good germination percentage and vigor index value. The insignificant result in presence of molybdenum or zinc ion in absence of SMS33 or SKPS32 also proves that the molybdenum or zinc ion only shows its catalytic effect in presence of bacteria i.e. SMS33 or SKPS32. The *F* value statistics again signifies the outcome of the experiment result. Hence, this metal-bacteria combination also produce the healthy fruits within the lesser time than did the control. In this experiment, non-sterilized soil, water, and seeds have been used and overall outcome give significant outcome implies about our next to field trial in agricultural land.

5. Conclusion

It has been found from the experimental results that insoluble organic phosphate compound present in the soil can be degraded by bacteria catalyzed by suitable metal ion. In this experiment, we have used three non-pathogenic bacteria SMS33 strain, SPS32 strain, and SKPS41 strain and three metal ions such as Mo, Mn, and Zn for the degradation of soil lecithin. Among the various combinations of metal and bacteria PVK + L + AMo + SMS33 is the best combination for the complete biodegradation of lecithin into soluble phosphate compounds and many others valuable chemical (non-toxic) compounds. The degraded products produce *Abelmoschus esculentus* fruits at a faster rate than that produced by conventional methods. These metal ions activate the bacteria for the secretion of phospholipase enzymes and other valuable ecofriendly compounds. These metal ions are also plant nutrients. The bacteria can also consume excess metal ions from soil and water to reduce their toxic level. So the selective combination of metal and bacteria can be utilized for the degradation of insoluble compounds of the soil for balance of nature and its development.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement

We are thankful to Ms. Sreyasree Mandal for manuscript-proof-reading. We are also grateful to Dr. Achintya Mondal for arrangement of LC-MS analysis and analysis of LC-MS results. Furthermore, I am thankful to Mr. Manas Mandal, Mr. Subrata Maikap, and Mr. Partha Pratim Bose for their laboratorial assistance and sample collection.

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