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Original paper

Optimization of Myo-inositol hexakisphosphate phosphohydrolase (MIPH) production and enhancement of hydrocarbons degradation by purified MIPH

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Abstract

Objective: The present study characterizes bacteria with biodegradation ability and optimize the growth conditions of Myo-inositol hexakisphosphate phosphohydrolase (MIPH).

Methods: Out of the 15 bacterial isolates, one strain SM01 showed the highest MIPH activity was selected based on the diameter of halo zone formation on calcium phytate plates. SM01 strain was identified as a *Serratia marcescens* with the highest phytate activity which was further confirmed by partial sequencing of the 16S rRNA gene.

Results: MIPH enzyme was purified from Serratia strain which was found to exhibit a highly-specific MIPH activity and high specificity to the phytic acid but negligible activity against the other substrates tested. The purified MIPH enzyme had an isoelectric point of 6.8 and Molecular weight to 60 kDa. The degradative ability *of Serratia marscescens* shown that both the culture and the purified enzyme had maximum MIPH activity at the 36 h of incubation. 0.5 U/ml concentration of the purified MIPH enzyme was found to show similar activity as the 10^8cfu/ml culture tested.

Conclusion: Serratia marcescens SM01 strain was capable of degrading hydrocarbons due to their varied strategies to produce the MIPH enzyme. This could be made useful in the biodegradation of polluted soils and the environment.

Keywords *Serratia marscescens*, myo-inositol hexakisphosphate phosphohydrolase (MIPH), Oil degradation, phytic acid.

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Introduction

Myo-inositol hexakisphosphate phosphohydrolase is the major form of phosphorous storage in soils which is present as complex organic matter due to its strong binding capacity to mono or divalent cations such as Ca, Mg, Fe, Cu, etc. (CERINO et al, 2012; SELLE et al, 2012; SHIM and OH, 2012); and therefore remains unavailable for plants (LAZALI et al, 2013; MITTAL et al, 2012; SHIVANGE et al, 2012). Myo-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases are a group of catalyzing enzymes which hydrolyses phosphomonoester bonds in phytate, thereby releases lower forms of myo-inositol phosphates and inorganic phosphate which are readily available for further use of plants (SHIN et al, 2001; SHIVANGE et al, 2010). MIPHs, are a diverse group of enzymes classified based on the catalytic mechanism such as histidine acid MIPHs (HAPhy), ß-propeller MIPHs (BPPhy), cysteine MIPHs (CPhy) and/or purple acid MIPHs (PAPhy). Based on pH optimum, MIPHs is also called acid or alkaline MIPHs and based on the carbon in the myo-inositol ring of phytate at which dephosphorylation is initiated, MIPHs are classified into 1/3-MIPHs, 4/6-MIPHs and 5-MIPHs (GREINER, 2006; MULLANEY and ULLAH, 2003).

It is mainly present in soil, plants, and microorganisms. Most importantly, microorganisms produce MIPHs extracellular secreted into the soil for plant debris decomposition and liberation of the phosphorous from organic compounds, thus play a highly significant role in the minerals cycling in the soil. 3-MIPHs and Purple acid phosphatases (PAPs) are widely found in fungi, bacteria, and plants. Previously published reports have highlighted the possible role of soil microbes in the release of phosphorous. Bio-degrading organisms include aerobic bacteria, anaerobic bacteria, and fungi. MIPH activity is most frequently found in Aspergillus species including A. terreus, A. ¢cuum, and A. niger, Aerobacter aerogenes, Pseudomonas sp., Escherichia coli, Bacillus subtilis, and the MIPH-encoding genes have been described in various bacterial species such as *Aerobacter aerogenes*, B. *amyloliquefaciens*, *Bacillus subtilis*, *Klebsiella* sp., *Pseudomonas* sp., *Enterobacter* sp., *Escherichia coli*, *Selenomonas ruminantium* and *Bacteroides multiacidus* (YOUNG et al, 1998; DAI et al, 2011; ALIYA et al, 2015). The microbial MIPHs have various advantage as they are cheaper and less time consuming in enzyme production. Hence, search for microbial source of novel and efficient MIPHs are increased in recent years due to its potential biotechnological applications (KUMAR et al, 2013; YANKE et al, 1999; YOON et al, 1996; HARPREET et al, 2016). Since there are only a few reports available on MIPH producing bacteria, the present study was performed to isolate, characterize and optimize the growth conditions of MIPH producing bacteria from soils thatcan be developed for biodegradation of hydrocarbons.

Materials and Methods

Collection of samples and characterization of bacteria

The MIPH solubilizing bacteria were isolated from 25 soil samples collected different petrol stations in and around the Riyadh region. Topsoil was collected by spatula into clean sterile plastic bags and stored at 4°C before use. The soil samples were air-dried (20°C) and passed through a sieve (mesh size, 2 mm).

Isolation, screening, and selection of bacterial isolates

The bacterial strains were isolated as previously described (Kumar et al. 2013) by serial dilution techniques and 100 µl of the final dilution were plated onto MIPH screening media containing PSM; 10 g l-1 D-glucose, 4 g l-1 Ca-phytate, 2 g l-1 NH4NO3, 0.5 g l-1 KCl, 0.5 g l-1 MgSO4.7H2O, 0.01 g l-1 FeSO4.7H2O, 0.01 g l-1 MnSO4.H2O, 15 g l-1 Agar. The plates were incubated 37ºC for 24-48 hrs (KEROVUO et al, 1998) and then examined for halo zone around bacterial culture on calcium phytate plates. The isolated bacteria were grouped into various genera as per Bergey's Manual of Determinative Bacteriology (GREINER et al, 2006) and were characterized based on their morphology, Gram staining, spore staining, motility, oxidase, catalase, oxidation fermentation, gas production, ammonia formation, nitrate and nitrite reduction, indole production test, methyl-red and Voges–Proskauer test, citrate, and mannitol utilization test, hydrolysis of casein, gelatin, starch, urea, and lipid. *Bacillus* sp. *Micrococcus* sp. *Vibrio* sp. *Pseudomonas* sp. *Thiobacillus* sp., *Xanthobacter* sp., *Serratia* sp., *Enterobacter* sp., and Acinetobacter sp. were identified from the 25 soil samples. The isolates forming hydrolysis zones were purified for their phytate hydrolyzing potential and pure cultures of MIPH positive bacterial isolates were maintained on LB agar plates and also stored as glycerol stocks. One isolate which showed comparatively high MIPH activity and solubilization index, identified as Serratia marcescens strain was selected for further purification and biodegradation studies. Molecular biology and analytical grade chemicals procured from Sigma, Merck, and Hi-Media Pvt. Ltd was used for all the studies.

Purification of the enzyme

The *Serratia marcescens* strain with high MIPH activity was grown in Luria broth supplemented with phytate. The samples were withdrawn from the culture media at different time points, centrifuged, and then passed through a PD-10 gel filtration column (Pharmacia Inc., Uppsala, Sweden). The crude enzyme preparations were purified by adding $CaCl_2$ to a final concentration of 1 mM and the enzyme was precipitated by adding 3 volumes of cold (220°C) ethanol with constant stirring overnight. The precipitate was collected and washed with cold (220°C) ethanol and once with cold (220°C) acetone and dried by lyophilization. The dried precipitate was saturated with ammonium sulfate twice to give 85% saturation and

the final precipitate was dissolved in 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM CaCl2 and stored at 220°C. For enzyme assays, the frozen enzyme preparations were thawed and passed through a PD-10 gel filtration column (Pharmacia) into an appropriate buffer and used. The isoelectric point and the molecular weight of the enzymes were determined by Phast Gel IEF 3-9 isoelectric focusing gels (Pharmacia) and one dimensional SDS-PAGE respectively. An unstained broad range protein standard ranging from 14 to 116 kDa was used for the detection of the molecular weight of MIPH in this study.

Protein identification

Proteins in the purified enzyme MIPH of SM01 strain preparations were identified using LC-MS/MS method. The electrospray mass spectra were performed using Agilent 1100 liquid LC system (Santa Clara, CA, USA) coupled to a Quattro Ultima triple mass spectrometer (Micromass). MS/MS raw data were used to search against the protein database at NCBI (http://www.ncbi.nlm.nih.gov) and the data were analysed using Agilent Ion Trap Analysis software version 5.2 and proteins were identified by database search against the MASCOT database.

Estimation of degradation activity

The activity was determined by a method described by Engelen et al. 1994. Briefly, reaction mixture consisting of 100 µl of enzyme sample and 900 µl of 0.1 M acetate buffer, pH 6.0, 500 µl of 5 mM sodium phytate in 0.1 M acetate buffer, pH 6.0 as substrate were and incubated at 37ºC for 30 min. The reaction was stopped later by adding 500 µl freshly prepared color reagent. A blank reaction was maintained for the procedure wherein a color reagent was added before before the incubation and the substrate solution was added after incubation. The color developed from the MIPH activity was determined at 415 nm. One unit MIPH was defined as the amount of enzyme that released 1 µM of inorganic phosphate in 1 min. The amount of phosphorus released was calculated based on the standard curve of KH2PO4.

Effect of pH on the activity

The effect of pH on the activity of the SM01 strain MIPH enzyme was assayed as described by Gulati et al, 2007. Briefly, the optimal pH of the MIPH activity from Serratia marcescens SM01 was determined by measuring the activity pH range 4.0-8.0, using 0.1 M acetate (pH 4.0-5.0), 0.1 M phosphate buffer (pH 6.0-7.0) and 0.1 M Tris (pH 8.0) buffers. Maximum activity was taken as the optimum pH for MIPH for activity expressed in comparison with maximum activity.

Effect of temperature on the activity

The buffer- enzyme mixture was incubated at various temperatures from 30-60°C to determine the effect of temperature on enzyme activity. At 65 min incubation period, a two ml color stop mix was added and the enzyme activity was measured according to the standard assay method at every 5ºC rise in temperature as described by Ekundayo and Osunia, 2013.

Substrate specificity

The substrate specificity of the enzyme from the SM01 strain was determined using the reaction mixture containing 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM CaCl2 and 2 mM tested substrate. 0.1 mg/ml pepsin and trypsin, ADP, ATP, p-Nitrophenyl Phosphate(p-NPP), Glucose-6-Phosphate, and fructose-6-phosphate were tested by incubation at 30ºC for 24 hours at pH 6.

General DNA techniques

DNA was extracted from all isolates following overnight growth bacteria suspended thoroughly in 1 ml distilled water and boiled in a water bath for 10 min. After centrifugation, the supernatant is used as a template DNA. PCR mixture of composed from 12.5 µl of GoTaq® Green Master Mix (2x) (Promega, USA), 5µl template DNA, 2 µl primers (for each) final concentration (0.6pmol/µl), and nuclease-free water up to 25 µl. The 16S rDNA sequencing using universal primers (518F: 50-CCAGCAGCCGCGGTA ATACG-30 and 800R: 50TACCAGGGTATCTAATCC-30) were used for species-level identification of the MIPH producing isolates (HARPREET et al. 2016).

The amplified product was purified using GFXTM PCR DNA and Gel Band Purification kit (Amersham Biosciences). The sequencing reaction was carried out using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). For the sequencing reaction, the Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin–Elmer) was employed. The obtained sequences were analyzed using BlastN with existing sequences in the NCBI database and then deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov/GenBank/submit.html).

Determination of rates of utilization of crude oil, diesel and engine oil

The bacterial strain *Serratia marcescens* was grown in batch cultures in 500 mL Erlenmeyer flasks containing 300 mL of Bushnell Haas broth (Minimal Salts medium) consisting of magnesium sulfate 0.20 g/L, calcium chloride 0.02 g/L, monopotassium phosphate 1 g/L, dipotassium phosphate 1 g/L, ammonium nitrate 1 g/L, ferric chloride 0.05 g/L, pH 7, supplemented with 1 g of 1% refined petroleum product (crude oil, diesel, and engine oil) as the only source of carbon. The ability of the *S.marcescens* strain to degrade the petroleum product and use them as a sole source of carbon directly was tested and compared with the purified MIPH enzyme's role in the same. To 100 ml of the broth 0.1 ml of 10^8 CFU/ml culture was inoculated, followed by the inoculation of 0.1 ml filter sterilized crude oil, diesel, and engine oil and incubated at 37ºC on a rotary shaker for three days. Three different concentrations of 0.5 U/ml, 1 U/mL, and 2 U/mL of the purified enzyme were tested against the 1% refined petroleum product for optimization and comparison.

At the end of the incubation period, the optical density of each flask was measured at 650 nm using spectronic 20 Genesys spectrophotometer, which is the index of growth reflecting the potential for the biodegradation of the petroleum products by the bacterial strain. Uninoculated

control flasks were maintained in all experiments in duplicates and purity of the test was analyzed in intervals by pour plate technique.

Results

In the present study, bacterial strains were isolated from a soil sample of area locating near to different petrol stations and screened on MIPH screening media plates. Initially, only 15 isolates were found forming zone of hydrolysis which were then purified and again grown on PSM plates to reconfirm their MIPH production ability (Figure 1). Out of the 15 bacterial isolates, one of the strains designated as SM01 showing the highest MIPH activity was selected based on the diameter of halo zone formation on calcium phytate plates. This SM01 strain showed a maximum diameter of zone of 3.8 cm and the enzyme activity was 0.385 U/ml which suggests the ability of the strain to use phytate as a substrate at 37ºC. Based on these properties, the SM01 strain was selected for further biochemical and molecular characterization studies.

Figure 1. PSM plates to reconfirm their MIPH production ability.

16S rDNA-based identification

Based on morphological and biochemical characteristics SM01 strain was identified as a Serratia marcescens with highest phytate activity which was further confirmed by partial sequencing of the 16S rRNA gene and BLAST analysis. The 16S rDNA sequence analysis showed 98% similarities with the Serratia sp. Strain (GenBank Accession No. KR133277) existing sequence in the NCBI database (Figure 2).

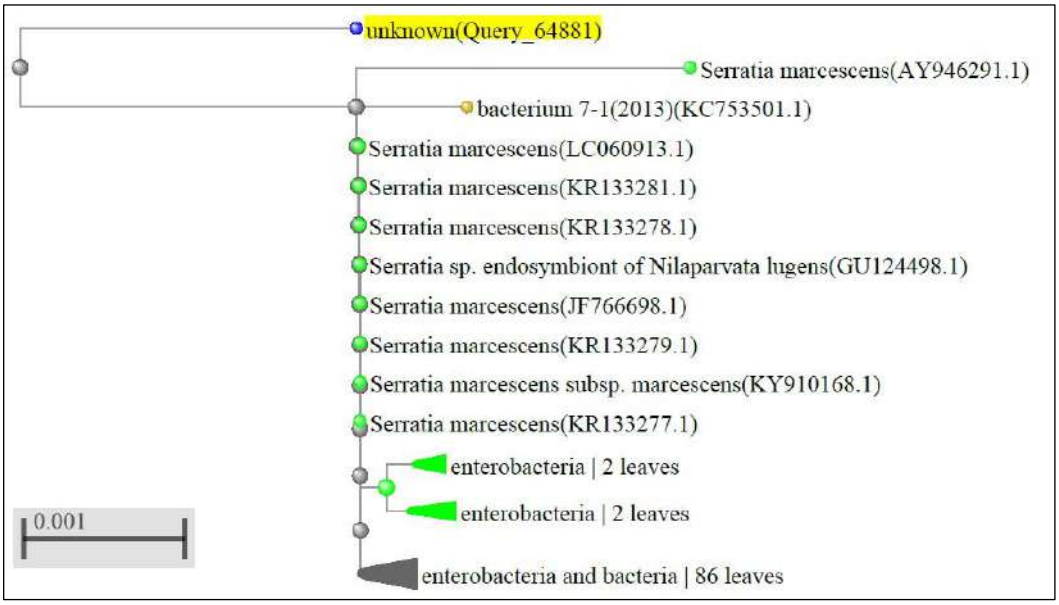

Figure 2. 16S rDNA-based identification-showed 98% similarities with the Serratia sp. Strain (GenBank Accession No. KR133277) existing sequence in NCBI database.

Purification of the MIPH enzyme

MIPH enzyme required CaCl2 in all its purification steps to maintain the enzyme activity and lose the activity if EDTA was used in buffers. A method that used combination of purification by ethanol and ammonium sulfate precipitation was considered to be the best method for enzyme purification and zation characterization. MIPH enzyme was purified from Serratia strain SM01 strain using PD-10 gel filtration column chromatography by $CaCl_2$ and ethanol selective precipitation followed by saturation with

ammonium sulfate. The purified MIPH protein had been stored in 100 mM Tris-HCl (pH 7.5) supplemented with 1 mM CaCl2, at 220°C. The purified enzymatic assay samples were used for the determination of MIPH activity and based on the analysis of released Pi using colorimetric phosphate analysis methods, the activity of SM01 has resulted in an activity of 152 mU/ml (Figure 3). The effect of pH and temperature on the MIPH activity of the isolate was assayed. The Serratia marscescens isolate had higher phytate activity at 40°C and was highly stable in broad pH range (pH 4-7) with optimal pH being 6.0 (Figure 4).

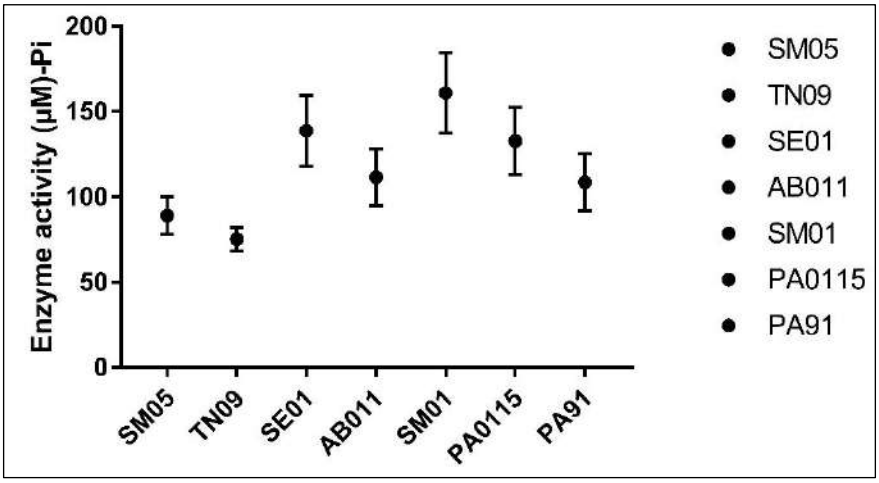

Figure 3. Activity of SM01 was resulted in an activity of 152 mU/ml.

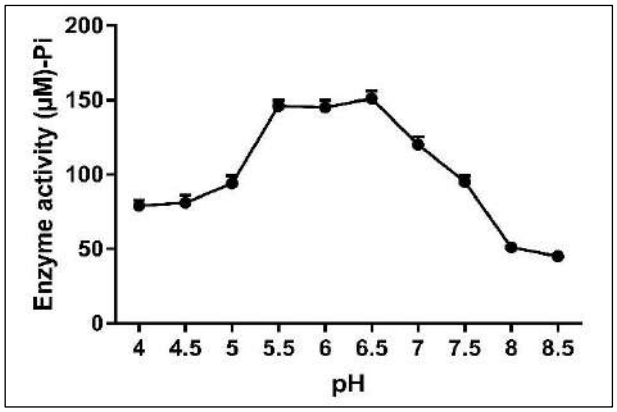

Figure 4. phytate activity at 40ºC and were highly stable in broad pH range (pH 4-7) with optimal pH being 6.0.

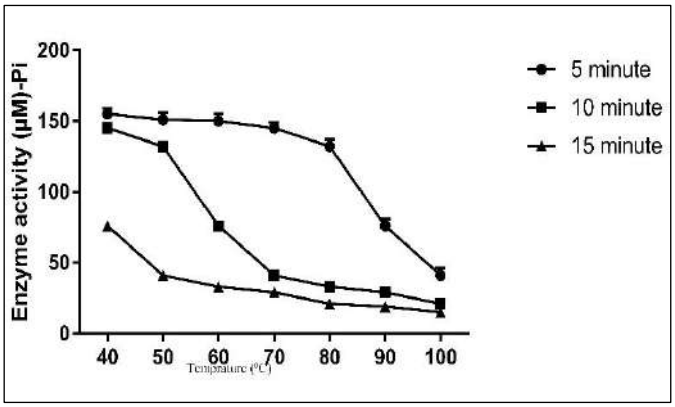

Figure 5. This purified protein was found to exhibit a highly- specific MIPH activity.

Moreover, a decrease in pH showed a decrease in MIPH activity. Even after treating at 80°C for 10 min, the strain maintains 80.5% of its initial activity, followed by 60.8% activity after treatment at the same temperature for 20 min (Figure 5). This purified protein was found to exhibit a highly-specific MIPH activity. The enzyme showed high specificity to the phytic acid but negligible activity against the other substrates tested (Figure 6).

The purified MIPH enzyme of SM01 strain was subjected to one dimensional SDS-PAGE and isoelectric focussing to determine the molecular weight and isoelectric point respectively. Only One large polypeptide band was detected by silver staining corresponding to 60 kDa and also the enzyme had an isoelectric point of 6.8 (Figure 7). LC-MS/MS spectrometry method was employed to identify the proteins in the purified MIPH preparations. The results showed that there were 4 different groups of proteins in the sample out of which one protein exhibited high MIPH activity according to NCBI protein database (http://www.ncbi.nlm.nih.gov) (data not are shown).

Degradation of crude oil, diesel and engine oil by the MIPHs

The ability of the *S. marcescens* strain to degrade the petroleum product and use them as a sole source of carbon directly was tested. The degradative ability of *Serratia marscescens* on crude oil, diesel, and engine oil are as shown in Figure 8. It was shown that both the culture and the purified enzyme of the SM01 strain had maximum MIPH activity at the 36 h of incubation. 0.5 U/ml concentration of the purified MIPH enzyme was found to show similar activity as the 10^8cfu/ml culture tested.

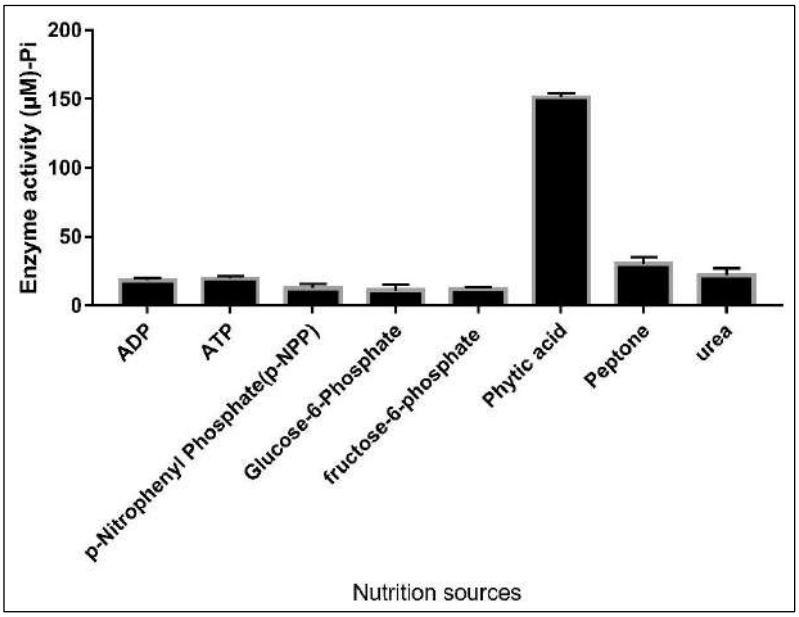

Figure 6. The enzyme showed high specificity to the phytic acid but negligible activity against the other substrates tested.

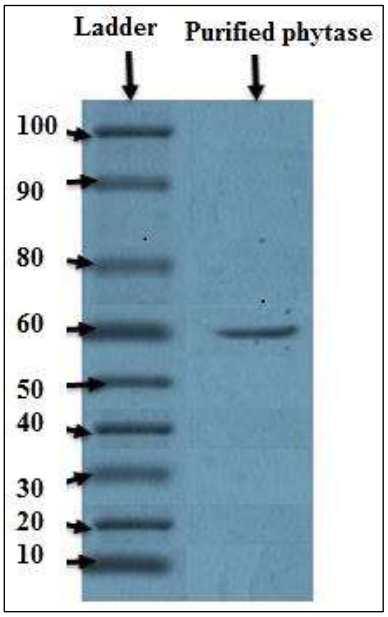

Figure 7. Only One large polypeptide band was detected by silver staining corresponding to 60 kDa and also the enzyme had an isoelectric point of 6.8.

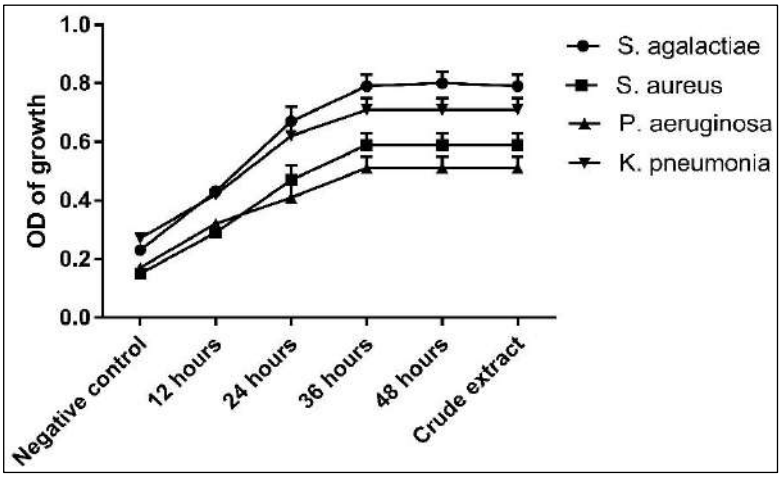

Figure 8. The degradative ability of *Serratia marscescens* on crude oil, diesel, and engine oil.

Discussion

MIPH enzyme known as Myo-inositol hexakis-phosphate phosphohydrolase hydrolyzes phytic acid to Myo-inositol and phosphoric acid in different steps to form myo-inositol phosphate intermediates. They catalyze phosphate monoester hydrolysis of phytic acid (*myo*-inositol hexakisphosphate), which results in the stepwise formation of *myo*-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphates, as well as the liberation of inorganic phosphate. Microbial MIPH is efficient in reducing the level of phosphates in the soil and improve phosphorous utilization by the plants (HARPREET et al, 2016; YOON et al, 1996; ENGELEN et al, 1994) Different sources of MIPHs have been identified in recent years of which microbial MIPHs have gained importance due to their varied advantages. In the present study, the MIPH producing bacteria were identified from soil taken from different petrol stations. We identified 15 isolates forming a zone of hydrolysis due to MIPH production which were then screened and purified on PSM plates. To further confirm the identity, the molecular characterization was carried out using 16S rRNA sequence homology. Based on molecular characterization, the isolate with high phytate hydrolyzing activity showed 98% similarity with *Serratia* sp. (GenBank Accession No. KR133277) strain available in NCBI GenBank library.

Serratia marcescens SM01 had shown the highest MIPH activity among the 15 isolates. This strain had an enzyme activity of 0.385 U/ml and was selected for further

biochemical and molecular characterization studies. Harpreet et al. 2016 identified degraded wood as a source of novel MIPH positive isolates and Pepi et al. 2010 identified degraded wood as a source of tannin degrading Serratia sp. This reveals the diversity of MIPH producing isolates in the environment and emerging novel microbes in MIPH production. The MIPH from Serratia marcescens SM01 investigated in this study was observed to have very good phytate hydrolyzing property with extracellular MIPH production and stability at varying temperatures and pH. The methods used in this study to screen the phytate activity using calcium phytate plates which were previously described in Bacillus sp. (KEROVUO et al, 1998; KIM et al, 1998; SHIMIZU, 1992), was proved to effectively identify the phytate producing isolates from the soil. Different MIPHs have been identified and purified from different microorganisms and plant sources. In this study we purified a MIPH enzyme from Serratia marcescens sp. and the MIPH activity was tested against a phytate substrate. The purified MIPH enzyme was subjected to one dimensional SDS-PAGE and isoelectric focussing to determine the molecular weight and isoelectric point respectively. Only One large polypeptide band was detected by silver staining corresponding to 60 kDa and also the enzyme had an isoelectric point of 6.8.

LC-MS/MS spectrometry method, employed to identify the proteins in the purified MIPH preparations, showed that there were 4 different groups of proteins in the sample. Only one group of protein exhibited high MIPH activity according to NCBI protein database in this study and was used for further testing. In contrast. Dai et al., 2011, reported eleven different proteins in the purified MIPH sample and observed MIPH activity in one single protein later identified as Purple Acid Phosphatases. Characterization of the purified MIPH enzyme was done to identify their ability to solubilize different phosphate compounds. The purified MIPH from the SM01 strain was thus found to exhibit a highly-specific MIPH activity to phytic acid at optimum pH and temperature but negligible activity against the other substrates tested. The MIPHs from SM01 isolate had higher phytate activity at 40°C and were highly stable in a broad pH range (pH 4-7) with optimal pH being 6.0. The optimum pH of this enzyme falls in the common range of pH 4.5-7.5 for the activity of other bacterial MIPHs as identified previously (KEROVUO et al, 1998; KIM et al, 1998). A previous study reported more than 80% residual MIPH activity after 65-95ºC treatment withholding times from 1 to 10 min, 81% of its original activity at 80°C for 10 min, and 70% activity up to 20 min at 80°C with excellent tolerance and retention. Similar results were identified in our study with varying temperature stability viz., 80°C for 10 min, with 80.5% of its initial activity, followed by 60.8% activity at 80°C for 20 min which is found to be more stable than other commercially available MIPHs. The optimal temperature for MIPH from SM01 isolate was higher than that of the optimal temperature of MIPH from Bacillus sp. KHU-10 (CHOI et al, 2001; SHIM et al, 2012) but lower than Bacillus sp. DS11 and *Klebseilla aerogenes* (JAREONKITMONGKOL et al, 1997; KIM et al, 1998). Harpreet et al. 2016 identified MIPHs with higher optimum temperature due to the thermostability of the strain Enterobacter cloacae strain PSB-45 but comparatively lesser than the PSB-15 Serratia strain. Thus, the above properties suggest the MIPH enzyme isolated in this study is highly suitable for commercial production of the enzyme.

In this study, the ability of the *S. marcescens* strain to degrade the petroleum product and use them as a sole source of carbon directly was also tested. *S. marcescens* SM01 isolated in this study showed evidence of a high ability to degrade crude oil as well as diesel and engine oil. The highest degradative ability by this isolate on all types of hydrocarbon was observed after 48 hours of incubation. Moreover, the rapid degradation of crude oil and engine oil was observed while the slow degradation of diesel at all times of incubation was identified. Both the culture (10^8cfu/ml) and the purified enzyme (0.5 U/ml) had maximum MIPH activity at the 36 h of incubation. This characteristic of the SM01 MIPH has to be more studied to be used as a potential enzyme for biodegradation but previously published reports suggests that this ability in other microbes may be attributed to the presence of saturated alkane with intermediate chain (C10 – C24) length (CHOI et al, 2001). Many reports identified bacterial isolates which showed high diversity and adaptability in the utilization of different organic molecules as a carbon source and also their degradative ability on a specific hydrocarbon as a source of energy and/or biomass may differ (JAREONKITMONGKOL et al, 1997; KIM et al, 1998). But our results suggest the high specificity of the MIPH enzyme but also a significant activity with the hydrocarbons tested. Phosphorus is known to be one of the limiting nutrients in the biodegradation process. The ability of a bacteria to degrade a hydrocarbon in polluted soil is improved by the presence of phosphorus which is readily made available by the hydrolyzing effect of MIPH on the complex salts of phosphorus that are insoluble in the soil (KIM et al, 1998). Moreover, microbes used certain strategies to degrade hydrocarbon such as the insertion of O_2 into the carbon chain through oxygeneases enzymes (YOUNG et al, 1998). Thus, aerobic conditions are therefore essential for microbial oxidation of hydrocarbons in the environment.

Conclusion

It was concluded that the isolated Serratia marcescens SM01 strain was capable of degrading the hydrocarbons due to their varied strategies to produce MIPH enzyme and also to tolerate elevated hydrocarbon concentrations. This could be made useful in the biodegradation of polluted soils and the environment. The isolated SM01 strain utilized crude oil, diesel, and engine oil as the sole source of carbon and energy and degraded them in optimum laboratory conditions. Thus concluding the potential role of MIPH producing soil bacteria in the bioremediation of polluted ecosystems.

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