3.1 Chemicals

Technical grade monocrotophos (76% EC) was obtained locally from Ahmedabad, India. Stock solution and working solutions were prepared freshly in distilled water. AR grade chemicals were used throughout the study.

3.2 Isolation and screening of monocrotophos degrading bacteria

3.2.1 Sample collection:

Five soil samples for isolation of monocrotophos degrading bacterial strains, were collected from different fields of Gandhinagar district of Gujarat used to cultivate various crops like vegetables, cotton etc. These fields have been exposed to organophosphorus pesticides including monocrotophos for several years. The soil samples were collected from depth of 15 cm. The collected soil samples were mixed properly, sealed in sterile plastic bags and preserved at 2^0C until further analysis.

3.2.2 Enrichment and isolation of soil samples:

For enrichment of soil samples to obtain pesticide degrading bacterial strains, 10 gm of each soil sample was put aseptically in sterile conical flask and 10 mL of 100 ppm monocrotophos (MCP) solution was added in it and mixed properly by sterile glass rod. The flask was kept at room temperature in static conditions for five days. After incubation, soil suspensions were prepared in sterile distilled water by serial dilution method from these soil samples. 1 mL aliquots from 10^{-1} to 10^{-3} dilution were inoculated on nutrient agar plate containing 100 ppm MCP by spread plate technique for all the soil samples. Plates were incubated for 24 hours in incubator at 37^0C. After incubation well isolated colonies were further inoculated on Nutrient agar plates containing 100 ppm MCP by streak plate method to obtain pure colonies. These plates were incubated for 24 hours in incubator at 37^0C. Colony characteristics of these pure bacterial colonies were observed and microscopic study of these colonies was done. These colonies were then inoculated on Nutrient agar medium containing 250 ppm MCP and incubated at 37^0C for 24 hours. After incubation, the colonies obtained were then transferred to 500 ppm MCP containing Nutrient agar plates and incubated as stated earlier. This process was then repeated with Nutrient agar medium having 700, 1000 and 1500 ppm MCP concentration. Finally, the bacterial colonies obtained on 1000 ppm MCP containing Nutrient agar

medium were further used for screening procedure and maintained on Nutrient agar slants.

3.2.3 Screening of monocrotophos (MCP) degrading bacteria:

For screening of potent MCP degrading bacteria, isolated bacterial cultures were inoculated in mineral salt medium (MSM). Growth of isolated pure bacterial cultures maintained on Nutrient agar slant above was scrapped by sterile wire loop in presence of 1mL sterile distilled water. Obtained sterile distilled water suspension of culture was subjected to spectrophotometer to read its absorbance at 600nm. The inoculum density was adjusted to OD_{600} = 1. From this culture suspension having OD value 1 at 600nm, 1mL of was inoculated aseptically in a flask containing 100 mL MSM broth having 1000 ppm MCP. After inoculation, all the flasks were incubated in shaking condition at 150 rpm at 37^0C for 7 days. Screening of potent MCP degrader form all the isolated cultures was done based on their ability to degrade the 1000 ppm MCP in MSM broth using HPLC analysis. To extract the MCP residues, culture broth obtained after incubation from all the flasks was centrifuged (REMI Heavy Duty Cooling Centrifuge) at 15,000 rpm at 4^0C for 15 minutes. The cell free supernatants obtained were then used to estimate the residual MCP present in it by high-performance liquid chromatography (HPLC).

3.2.4 HPLC analysis:

Degradation of pesticide was estimated on the basis of MCP residue determination from sample using HPLC with WATERS - 2489 UV detector which was having 250 mm x 4.6 mm stainless steel (5µm) C18 column. The mobile phase used was acetonitrile : water (20 : 80) were pumped from the solvent reservoir with a flow rate of 1.0 mL/min and 0.02 mL of samples were applied for this. HPLC was operated at ambient (28 ± 2^0C) temperature. MSM amended with 1000 ppm MCP without microbial inoculation was used as control and MCP residues were extracted by the similar procedure applied for test samples. Potential bacterial culture was screened out based on MCP removal ability and used for further studies. % degradation was calculated from the retention time and peak area of test sample corresponding to the standard and control samples.

3.3 Identification of bacterial isolate

3.3.1 PCR amplification and sequencing of 16S rRNA gene:

For identification of potent bacterial isolate named as KPA-1, 1 mL inoculum of potent culture was inoculated in 1000 ppm MCP containing 100 mL MSM medium and incubated at 150 rpm, 37^0C for 144 hours. After incubation, the culture broth was centrifuged at 15,000 rpm for 15 minutes at 4^0C. Obtained cell biomass was washed with sterile MSM broth and suspended in fresh, sterile, MSM broth and used for DNA extraction. For DNA isolation, bacterial DNA isolation kit Xcelgen was used. To check quality and purity of DNA, agarose gel electrophoresis was done using agarose 0.8% (w/v). The DNA was further used for PCR. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730x1 Genetic Analyzer.

8F: 5’ AGAGTTTGATCCTGGCTCAG 3’

1492R: 5’ ACGGTACCTTGTTACGACTT 3’

PCR was carried out in a final reaction volume of 25µL in 200µL capacity thin wall PCR tube in eppendorf Thermal Cycler. Composition of reaction mixture is given in **Table 3.1**. The PCR protocol designed for 30 cycles for the primers used is given in **Table 3.2**.

Table 3.1: Composition of reaction mixture for PCR

Components	Quantity	Final
DNase-RNase free water	7.50 µL	-
2XPCR master mix	12.50 µL	1X
Forward primer	1.0 µL	10 pmole/ µL
Reverse primer	1.0 µL	10 pmole/ µL
Diluted DNA (30ng/)	3.0 µL	-
Grand total	25.0 µL	-

Table 3.2: Steps and conditions of thermal cycling for PCR

Steps	Temperature	Time	Cycles
Initial denaturation	96^0C	2min	1
Final denaturation	94^0C	30sec	30
Annealing	52^0C	30sec	30
Extension	72^0C	90sec	30
Final extension	72^0C	10min	1

To confirm targeted PCR amplification, 5 µL of PCR product was electrophoresized on 1.2% agarose gel containing ethidium bromide (1%, 10 µL/mL). Then the amplified product was purified using Qiagen Minielute Gel Extraction kit. After that sequencing of the product was done.

3.3.2 Identification and phylogenetic analysis of the isolate

The sequence obtained was used to carry out the BLAST with the non-redundant (nr) data base of NCBI GenBank database to compare the sequence with those available in Genbank. For phylogenetic analysis, multiple alignments were carried out using Clustal W software. Phylogenetic tree was constructed using MEGA 4 (Tamura et al., 2011) and the distance metrix was generated using RDP databasefollowing Kimura 2 parameter method (Kimura, 1980).

3.4 Biodegradation of MCP by potent degrader

For the degradation study of MCP by potent degrader, the bacterial cell mass was harvested from its MSM slant containing 1000 ppm MCP, with sterile distilled water. Inoculum density of 1 mL inoculum was adjusted to $O.D_{600}$ = 1. Now 1 mL of such inoculum was inoculated in 250 mL flask containing 100 mL MSM supplemented with 1000 ppm MCP and incubated at 37^0C at 150 rpm for seven days. Samples were withdrawn at regular interval of 24 hours for seven days. These samples were centrifuged at 10,000 rpm for 15 minutes at 4^0C. Supernatant obtained was then used to estimate residual MCP by HPLC analysis. The conditions for HPLC were kept same as stated earlier.

3.5 **Real-time quantitative PCR for relative gene expression of organophosphorus degrading gene from bacterial isolate:**

To confirm the expression of opd gene in case of potent MCP degrader, expression analysis was done using RT q-PCR. For that, again the bacterium was inoculated in 1000 ppm MCP containing 100 mL MSM broth at 1mL inoculum density (OD_{600}=1) and incubated at 150 rpm, 37^0C for 144 hours. One sample was collected initially after inoculation and named as K1 which was served as control. While, another sample was collected after 144 hours incubation and tagged as K2 that served as test. Both the samples were centrifuged at 10,000 rpm, 4^0C for 15 minutes and the cell mass was washed with fresh and sterile MSM broth. Then the cell biomass was suspended in normal saline solution and used for further study.

Quantitative and qualitative analysis of total RNA:

Expression analysis of both the samples was carried out by two step method. Total RNA was isolated from cells pellet of sample 0 h and 144 hrs using TRIZOL® Reagent (Life Technologies) as per manufacturer's protocol.

Quantitative and qualitative analysis of total RNA:

Quantification of total RNA was done using Nano Drop 8000 spectrophotometer. Further, the quality of total RNA was analyzed on 1% denaturing agarose gel. 1 gm of agarose gel was dissolved in 36.25 mL DEPC (diethylpyrocarbonate) treated water, heated and then cooled to 60^0C. 5 mL of 10 X MOPS (3-(N-morpholino) propanesulfonic acid) buffer was added followed by 8.75 mL of 37% formaldehyde and then mixed properly and casted in the gel electrophoresis unit. For sample preparation, 2 µL RNA samples were mixed with 2 µL RNA loadig dye, heated at 70^0C for 5 minutes, chilled on ice for further 5 minutes and mixed with 0.5 µ EtBr (ethydium bromide) and loaded on the gel. Electrophoresis was carried out using 1X MOPS buffer and the gel image was captured using ChemiDoc XRS instrument (Bio-Rad).

cDNA synthesis:

Complementary DNA (cDNA) was prepared from the total RNA samples using H minus First Strand cDNA synthesis kit (Thermo Scientific) using random hexamer primer as per manufacturer's instruction. The reaction mixture composed of total RNA template 4µl, primers 1µl, DEPC-treated water 7µl, 5X reaction buffer 4µl, RiboLock RNase inhibitor (20 U/µl) 1µl, 10 mM dNTP mix 2µl and M-MuLV Reverse Transcriptase (20 U/µl) 1µl. After mixing gently, it was centrifuged and incubated for 5 min at 25^0C followed by 60 minutes at 42^0C. The reaction was terminated by heating at 70^0C for 5 min with temperature and then hold at 4^0C. The prepared cDNA samples were amplified using gene specific primers.

Primer design and synthesis:

LightCycler® Probe Design Software 2.0 was used for designing the primers to 16S rDNA gene (housekeeping) and organophosphorus degrading genes and then synthesized using Biolytic Dr. Oligo 96/192 synthesizer. Different primers corresponds to different genes which are responsible for the hydrolysis of organophosphorus compounds were tried for this purpose as listed in **Table 3.3**.

16S F (House keeping gene): 5' caatattccccactgctgcc 3'
16S R (House keeping gene): 5' cacctaggcgacgatccct 3'

PCR amplification of 16S and opdA gene:

The reaction mixture composed of nuclease free water 9.0 µl, cDNA 1µl, primers (10 pmole/µl) 1.0µl and 2X PCR master mix (MBI Fermentas) 13µl. Denaturation and annealing were carried out at 95^0C and 58^0C for 30 sec, respectively. Extension temperature was set as 72^0C for 45 seconds and final extensionwas 75^0C for 7 min in 40 cycles. Further the PCR products were quatified by 2% agarose gel electrophoresis. For that purpose, 1 g of agarose was heated in 50 mL 1X TAE (Tris-Acetate-EDTA) and cooled at 60^0C and 1% ethidium bromide was added at concentration of 10 µL/100 mL. 5 µL of PCR product was used for electrophoresis along with 100bp DNA molecular

weight marker (Thermo Scientific) and the electrophoresis was performed at 95 V for 30 minutes at room temperature.

Real Time PCR:

RT-qPCR reaction setup for both genes with two samples was performed in duplicate reaction using LightCycler 480 SYBR Green l Master (2X). Nuclease free water 9.5µl, cDNA 1µl, primers (10 pmole/µl) 1µl and LightCycler 480 SYBR Green l Master 12.5µl were included in the reaction mixture. PCR product was electrophoresed along with 100bp DNA molecular weight marker (Thermo Scientific). Electrophoresis was carried out at 95V for 30 min.

Table 3.3 Primers used for the detection of organophosphorus pesticide degrading genes

Gene	Primer	Sequence	Reference
opdA	Opda-F	5' tgttccggtaaccactcaca 3'	Horne et al., (2002)
	Opda-R	5' cactctcagagggacgaagg 3'	
opd	Opd-F	5' agggttgtgctcaagtctgc 3'	Mulbry et al., (1986)
	Opd-R	5' caataaactgacgtcgcgac 3'	
mpd	Mpd-F	5' agcaggtcgacgagatctac 3'	Zhongli et al., (2001)
	Mpd-R	5' ttgacgaccgagtagttcac 3'	
ophB	Ophb-F	5'cgtcgtcggctgggcagggt 3'	Barman et al., (2014)
	Ophb-R	5'gcgtgcggcctacctcgttg 3'	

3.6 Growth pattern of *Bacillus subtilis* KPA-1 in presence of MCP

To investigate the growth pattern of potent bacterial culture in presence of monocrotophos as carbon and energy source, 1 mL culture of potent bacterium at optical density of OD_{600} = 1was inoculated in MSM broth containing 1000 ppm MCP and incubated at 37^0C, at 150 rpm for seven days. The samples were withdrawn after every 24 hours till seven days and the samples were analyzed for bacterial growth which was measured spectrophotometrically (Systronics 169) at 600 nm. Here, the uninoculated MSM broth having 1000 ppm MCP as used as blank. For biomass study, the samples

were centrifuged at 7000 rpm for 15 min at 4^0C (REMI cooling microfuge) and the supernatant was discarded. Eppendoff tubes containing the cell biomass were allowed to air dry and then weight of all the samples was measured by weighing balance (Sartorius CP323S).

3.7 Metabolites of monocrotophos degradation:

3.7.1 Estimation of released inorganic phosphorus:

Inorganic phosphorus is one of the end products of organophsphorus pesticide particularly, monocrotophos degradation pathway. Thus, the release of inorganic phosphorus into the medium after incubation ensures the degradation of monocrotophos. To estimate the inorganic phosphorus as breakdown product after MCP degradation, the bacterial cell mass was harvested from the MSM slant amended with 1000 ppm MCP with sterile distilled water. O.D. of the inoculums was adjusted to 1 at 600 nm and 1 mL of such inoculum was then inoculated in 100 mL MSM broth supplemented with 1000 ppm MCP and incubated at 37^0C at 150 rpm for seven days and the samples were withdrawn at regular interval of 24 hours for seven days. These samples were centrifuged at 7000 rpm for 15 minutes at 4^0C and the cell free supernatants (CFS) were used to detect phosphorus released by Molybdenum-Vanadate method (Greenberg *et al*, 1992). For estimation of phosphorus, 50 ppm standard solution of phosphorus was prepared using KH_2PO_4. Different concentrations of standard and test samples were added to ammonium vanadate-molybdate solution. Ammonium molybdate and ammonium vanadate were prepared separately and mixed just prior to use. After that, all the standard and test solutions were diluted and the development of yellow color was observed which was measured spectrophotopmetrically at 470 nm. Then a calibration curve was prepared for concentration of phosphorus against their corresponding absorbance and the amount of released phosphorus was estimated by plotting the absorbance of test samples on calibration curve. For estimation of phosphorus released by degradation of MCP alone, phosphorus sources were emitted from the growth medium.

3.7.2 Estimation of CO_2 produced after MCP degradation

To check the presence of CO_2 as metabolite, two sets of anaerobic bottles containing 100 mL MSM having 1000 ppm MCP were prepared. Bottles were sealed with butyl rubber stoppers and aluminum seals. One bottle was served as uninoculated control while other bottle was inoculated with isolated potent culture by sterile needle of syring. The inoculum density of 1 mL inoculum was OD_{600} = 1. After inoculation bottles were incubated for 144 hours at 37^0C at 150 rpm, the headspace of bottles were checked for presence of CO_2 by Gas Chromatography (Chemito, India). During this analysis, hydrogen : carbon dioxide (80 : 20) was used as standard. Hydrogen was used as carrier gas with flow rate of 30 mL/min. Porapak Q Colum was used for the study and temperature of column, injector and detector were maintained at 50, 60 and 70^0C respectively.

3.7.3 Identification of metabolites by TLC after degradation:

Six 250 mL flasks of 100 mL MSM broth having 1000 ppm MCP were inoculated with the 1 mL inoculum of isolated *B. subtilis* KPA-1. Different flasks were incubated for 24, 48, 72, 96, 120 and 144 hours at 37^0C, 150 rpm. After incubation, culture broth from all the flasks were centrifuged at 10,000 rpm, 4^0C for 15 minutes and cell free supernatants were collected. Cell free supernatant from each flask was then mixed with equal volume of ethyl acetate. Ethyl acetate was then evaporated at room temperature and the remaining residues were dissolved in minimum volume of ethyl acetate. Such samples were analyzed for metabolites by TLC analysis. Six samples incubated for different time periods were spotted on different TLC plates along with one 0 hr sample which was considered as control. Apart from that, standard methylamine was also spotted to verify the results. All the plates were incubated in solvent system. The solvent system used was composed of n-butanol: acetic acid: water, 64: 16: 2 (Stahl, 1969). The chromatograms developed were observed under UV light.

3.7.4 Estimation of ammonia production after degradation

Ammonia has been proved as one of the metabolites produced during MCP degradation. To detect the ammonia as one of the metabolites, 100 mL of modified Christensen's medium having 500 mg/L methyl amine and 100 mL Davis Mingioli's

medium having 500 mg/L methyl amine were inoculated with the *B. subtilis* KPA-1 and incubated at 37^0C under shaking condition at 150 rpm till the color change of the media was observed. Ammonium sulfate was not added in the medium to ensure that, the color change was due to formation of ammonia from methylamine not due to ammonium ions released from ammonium sulfate.

3.8 Optimization study for MCP degradation by potent isolate using OFAT approach:

Different parameters like, medium pH, incubation temperature, inoculums age, inoculum size and carbon source were optimized during this study to enhance the degradation of MCP by isolated potent culture. This study was carried out by OFAT (One Factor at A Time) approach, thus during optimization of one parameter, other parameters were kept constant.

3.8.1 Effect of pH:

To determine the optimum pH for MCP degradation, different 250 mL conical flasks having 100 mL MSM broth containing 1000 ppm MCP were prepared and the pH of medium was adjusted from 5, 6, 7, 8, 9 and 10 by 1N HCl and NaOH. The flasks were than autoclaved at 121^oC, 15 lbs pressure for 20 min. and then cooled to room temperature. These flasks were now inoculated aseptically by 1mL culture of *B. subtilis* KPA-1 and incubated at 37^0C, 150 rpm for 144 hrs.

3.8.2 Effect of incubation temperature:

For optimization of incubation temperature, different 250 mL capacity conical flasks having 100 mL MSM broth supplemented with 1000 ppm MCP were inoculated with 1 mL inoculum of potent culture having density of OD_{600} = 1 and each flask was incubated at different temperature *viz*. 25, 30, 35, 40, 45 and 50^0C under shaking condition at 150 rpm for 144 hrs.

3.8.3 Effect of inoculum age:

To evaluate the most favorable inoculum age, potent bacterial cultures were grown in MSM broth by incubating them for different time periods ranging from 24, 48, 72, 96, 120 to 144 separately. 1 mL inoculum of such 24, 48, 72, 96, 120 and 144 old cultures were inoculated into various 100 mL MSM broth containing 1000 ppm MCP containing 250 mL conical flasks and incubated at 37^0C for 144 hrs. Inoculum density of 1 mL inoculum was adjusted to OD_{600} = 1.

3.8.4 Effect of inoculum size:

To know the most suitable inoculum size of potent culture for maximum MCP degradation, different volumes of inoculum ranging from 1, 2, 3, 4 to 5 mL of isolated bacterial culture at inoculum density of OD_{600} = 1 were inoculated in five 250 mL capacity conical flasks having 100 mL MSM broth + 1000 ppm MCP followed by incubation at 37^0C, 150 rpm for 144 hrs.

3.8.5 Effect of additional carbon sources:

Additional carbon source can enhance the MCP degradation. So, to determine the most stimulating carbon source, 0.1% glucose, sucrose, mannose, fructose, cellobiose and cellulose were added in the 100 mL MSM broth supplemented with 1000 ppm MCP in different 250 mL conical flasks followed by autoclaving and after sterilization, medium in all the flasks were inoculated with 1 mL volume of isolated bacterial culture. All these were incubated for 144 hrs at 150 rpm at 37^oC.

After incubation, all the samples collected for each parameter were centrifuged at 10,000 rpm at 4^oC for 15 minutes and the supernatants obtained were analyzed for removal of MCP by HPLC analysis at the operating conditions stated earlier. Finally after determining optimum conditions for maximum MCP degradation, one 250 mL conical flask having 100 mL MSM broth and 1000 ppm MCP was prepared and incubated as per optimized conditions while, another 250 mL conical flask containing 100 mL MSM broth having 1000 ppm MCP was prepared and kept for incubation under unoptimized condition. After incubation, the percentage of degradation of MCP in both the cases,

culture broth were centrifuged at 15,000 rpm for 15 minutes at 4^{o}C and the supernatants collected were used for HPLC analysis as explained earlier.

3.9 Pot study:

3.9.1 Soil analysis:

Pot study was performed to evaluate the response of crop in presence of pesticide and after biodegradation of pesticide by *B. subtilis* KPA-1 in soil. *Vigna radiate* varity K-851 was selected as experimental crop. The farm soil (0-20 cm depth) was obtained locally from, Gandhinagar, Gujarat. The soil was properly mixed and homogenized and further analyzed for various physico-chemical properties like texture by hydrometer method (Bouyoucos, 1962), water holding capacity (Keen and Raczkowski, 1921), moisture content using gravimetric method), pH by pH meter, available nitrogen using alkaline potassium permanganate method (Subbiah and Asija, 1956), available phosphorus was extracted by sodium bicarbonate and estimated by Olsen method (Olsen et al, 1954), potassium and sodium was extracted with neutral ammonium acetate and estimated by flame photometer (Hanway and Hiedal, 1952; Richards, 1954) and total organic carbon was measured by titrimetric method (Walkley and Black, 1934).

For determination of soil pH, soil solutions were prepared of by mixing soil samples with distilled water in 1:25 proportion and allowed to settle down. Now pH of the soil suspension was measured using pH meter (Digital pH meter, Systronics MKVI).

To estimate the total organic carbon content of soil, soil samples were digested with chromic acid followed by addition of sulfuric acid to provide heat and dilute the sample. Organic matter present in the soil reduced the chromic acid but excessive chromic acid which did not reduced by organic matter was estimated by titration with ferrous ammonium (FAS) solution and finally the total organic matter content of soil was calculated by following formula. Blank was also performed without soil sample addition.

$$\% \text{ organic carbon in soil} = \frac{(x-y) \times 0.003 \times 100}{2 \times W}$$

Where,

x = volume of FAS needed for reducing 10 mL of dichromate (blank reading)

y = volume of FAS needed for reducing the excess of dichromate (test reading)

W = weight of soil in gram

In case of available nitrogen of soil, alkaline potassium permanganate was used as extracting agent. Here, soil samples were mixed with the excessive amount of alkaline $KMnO_4$ followed by distillation process. As a result, ammonia was produced which was absorbed in known volume of H_2SO_4 followed by titration against standard NaOH solution and methyl red was used as an indicator. The available nitrogen content was calculated using following formula:

$$\text{Available nitrogen (kg/hector)} = \frac{(x - y) \times 0.28 \times 2 \times 1000}{W}$$

Where,

x = volume of H_2SO_4,

y = volume of NaOH used for titration,

W = weight of soil taken in gram

Determination of available phosphorus content of soil was done by Olsen method in which available phosphorus present in soil was extracted by shaking it with $NaHCO_3$ solution. Molybdo-phosphoric acid is formed when ammonium molybdenum solution was added to it which further reduced to a blue colored compound in presence of a reducing agent $SnCl_2$. The intensity of this blue colored compound was then measured spectrophotometrically at 660nm and finally, the available phosphorus content present in soil was calculated by given formula. Standard curve was prepared using different aliquots of KH_2PO_4 working solution. Standard set was run as per the procedure for test sample without soil samples and the absorbance was measured spectrophotometrically at 660 nm. Phosphorus content in soil sample was determined from standard curve and finally calculated by formula given below:

$$\text{Available phosphorus (kg/hector)} = R \times \frac{x \times 2}{y \times z}$$

Where,

R = concentration of P obtained from standard graph

x = total volume of extract

y = volume of aliquot of sample

z = weight of soil taken (g)

Unlike, above stated macronutrients, sodium and potassium are very important exchangeable metallic cations. To determine the amount of these cations, they were extracted from soil using neutral solution of ammonium acetate which was used as an extracting agent and most suitable for flame photometery. For determination of potassium and sodium a known quantity of soil sample was taken in a conical flask to this neutral ammonium acetate was added followed by mixing for half an hour on shaker and then filtered through whatman no. 42 filter paper. The filtrate was then used for determination in potassium and sodium content using flame photometer (Elico CL 361). Standard solution of 10, 40 and 80 ppm were prepared using KCl and NaCl salts dissolved in distilled water.

Available sodium and potassium (kg/hector) = R × dilution factor × 2.24

Where,

R= reading of test sample on flame photometer,

Dilution factor = total volume of extractant / weight of soil taken (g)

Micronutrients present in soil were also estimated using Atomic Absorbance Spectrophotometer. (AAS) (ICAEC Electronics Corporation of India Limited). Minerals of soil were extracted by DTPA (Diethylene Triamine Penta Acetic acid) extraction method (Lindsay and Norvell, 1978) in which, known amount of soil samples were mixed with DTPA extractant. After that, the soil solutions were placed in shaking condition for 2 hrs. followed by filtration through Whatman filter paper 42. This filtrate was used for estimation of zinc, manganese, copper, lead, nickel and iron by AAS and standard solutions for all the metals were also prepared using $ZnCl_2$, $MnSO_4\ H_2O$, $CuSO_4\ H_2O$, $Pb(NO_3)_2$, $NiCl_2\ 6H_2O$, $CdSO_4\ 8H_2O$ and $FeSO_4\ H_2O$ respectively. Blank solution was also prepared using all the reagents except soil. Readings for all the test and blank

solutions were recorded from observed in AAS. Micronutrients present in soil were calculated by formula given below:

Available heavy metal in soil (ppm) = (C – Cb) × 2

Where,

C = concentration of heavy metal in test sample,

Cb = reading of blank solution,

Dilution factor = 2 (20/10 = 2)

Apart from chemical properties, several physical properties were also measured. Water holding capacity is a very important property of soil, which was measured by Keen and Raczkowski's box method. For that, the soil samples were dried in oven at 105^0C and sieved. After mixing them properly, they were filled into a circular brass box with perforated bottom which was covered with a filter paper. The box was placed in petri dish containing water and incubated overnight at room temperature. The samples were then dried in oven. During whole experiment the weight of box was measured at every step to calculate the WHC of soil. The water holding capacity of soil samples were calculated as per following formula:

$$\text{WHC of soil} = \frac{W3-W2-W4}{W2-W1} \times 100\ \%$$

Where,

W1 = weight of box + filter paper,

W2 = weight of box + dry soil,

W3 = weight of box + soil after moistening it,

W4 = average weight of filter paper obtained after moistening them

To determine the texture of soil, samples were mixed with H_2O_2 and a dispersing agent. H_2O_2 has been used to destroy the organic matter present in the soil. Samples were then allowed to stand for 15 minutes. After dispersion of soil, all the samples were taken into 1000 mL cylinder and the samples were inverted at least for 20 minutes. The cylinder was placed on flat surface and after 20 seconds hydrometer was placed in the

samples. After 40 seconds, the reading on hydrometer was recorded. After 2 hrs, hydrometer reading was recorded again. Temperature was also recorded at both 40 seconds and 2 hrs interval. Now, sand, silt and clay proportion of soil samples were calculated from hydrometer readings. Hydrometer reading taken at 2 hrs was considered as clay percentage; while, silt proportion was obtained by subtracting the 2 hrs reading from the reading at 40 seconds; whereas, sand percentage was calculated by subtracting the 40 seconds hydrometer reading from weight of soil samples taken. After estimation of sand, silt and clay amount in percentage, soil texture was determined using soil textural triangle.

Soil moisture is an important factor that plays critical role in oxidation-reduction reactions in soil. In gravimetric method moisture content of soil was then measured based on the difference between wet and dry weight of soil samples. For this purpose, weight of clean and dry vessel was measured and recorded as Mc. This vessel was filled with moist soil and weight was measured which was named as Mcms. Soil sample with the vessel was dried in oven at 105^0C temperature till constant weight of the soil samples was achieved and the weight was recorded as Mcds and the moisture content of soil was calculated from given formula:

The water content (W) (%) = $\frac{\text{Mw}}{\text{Ms}}$ x 100

Where,

Ms = Mcds- Mc

Mw = Mcms-Mcds

3.9.2 Experimental setup and treatments application:

Three treatments were framed in this experiment viz. control, soil containing monocrotophos with application of growth suspension of *Bacillus subtilis* KPA-1 and soil containing monocrotophos alone without microbial culture. Each treatment was replicated five times. Thus a total of fifteen clay pots of 30 X 28 cm^2 were filled with 10 kg homogenized farm soil and arranged by randomization technique following random number table (Gomez and Gomez, 1983). Now 20 mL Technical grade monocrotophos (76% EC) was mixed with 100 mL distilled water and added in soils except control to

give its final concentration of 1000ppm. Culture of *Bacillus subtilis* KPA-1 was grown aseptically in laboratory in shake flask culture using mineral salt medium (K_2HPO_4: 1.5 g/L; KH_2PO_4: 0.5 g/L; $MgSO_4$: 0.2 g/L; $(NH_4)_2SO_4$: 0.5 g/L; NaCl: 0.5 g/L; $FeSO_4$: 0.02 g/L; $CaCl_2$: 0.05 g/L; supplemented with 1000 ppm MCP of pH: 7.0). After incubation as per optimized conditions, the cells were harvested by centrifugation at 15,000 rpm for 15 minutes at 4^0C and mixed with 1L sterile distilled water and added in soils of respective treatment and mixed well. After two days ten seeds of green gram [*Vigna radiate* (L.)] variety K-851, procured from Gujarat State Seeds Corporation Limited, Gandhinagar, Gujarat, were sown at 1.0 cm depth in each pot. The pots were hand weeded and watered as and when required. Different soil and plant parameters were analyzed at two different stages i.e. at 40 DAS (Days After Sowing) and 65 DAS.

3.9.3 Growth and yield parametes of Vigna radiatae (L.):

Germination percentage in each treatment was recorded at 6^{th} day using the following formula,

$$\text{Germination percentage} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}} \times 100$$

Thinning was done at 10^{th} day and 7 plants were raised upto 40 DAS. Three plants from each pot were uprooted at 40 DAS keeping four plants to grow upto yield level i.e. 65 DAS. Parameters like fresh and dry weight of plant, number and fresh and dry weight of nodules, fresh and dry weight of shoot and root were estimated at 40 and 65 DAS; and number and weight of pods per plant and number and test weight of seeds were measured at 65 DAS. Fresh weight of plants, root shoot, nodules, pods and seeds was measured immediately after uprooting the plants at 40 and 65 DAS.

3.9.4 Statistical analysis:

The experiment was laid down in Completely Randomized Block Design. Critical difference at 1 and 5% levels was calculated to find out the significance of treatments (Gomez and Gomez, 1983). Karl Pearson's Correlation Coefficient was also calculated between growth and yield parameters studied at 40 and 65 DAS (Rangaswamy, 2010).

3.9.5 Degradation of MCP in soil:

To check the degradation of MCP in soil samples at 40 and 65 DAS, 25 g soil samples were taken in 250 mL conical flasks. The residual MCP from each soil sample was extracted with stepwise addition of 30, 20 and 10 mL chloroform. Every time, the soil samples were transferred to separating funnel after addition of chloroform and allowed to separate them. After separation, the chloroform fractions were pooled out and the soil was again mixed with chloroform. The chloromorm fractions were mixed and allowed to evaporate at room temperature. After evaporation of choloroform, residual MCP was redissolved in minimum volume of ethyl acetate and residual MCP was then quantified by high-performance liquid chromatography (HPLC) with WATERS 2489 UV detector using 250 mm×4.6 mm stainless steel (5 μm) C18 column. Acetonitrile: water (20: 80) was used as a mobile phase at a flow rate of 1 mL/min.

3.9.6 Enzymatic study of soils:

Apart from that, enzymatic parameters like urease, acid phosphatase and alkaline phosphatase of soils for each treatment were estimated.

Acid and alkaline phoaphatase activities were estimated as per method of Tabatabai and Bremmer, (1969) and Eivazi and Tabatabai, (1977). Here released p-nitrophenol (PNP) is measured at 6.5 for acidic and 11 pH for alkaline phosphatase. Soil samples were mixed with modified universal buffer having 6.5 and 11 pH for acidic and alkaline phosphatase assays respectively, p-nitrophenyl phosphate and toluene followed by incubation at 37^0C for 1h. After incubation solutions of $CaCl_2$ and NaOH were added in it and mixed well. Now the content was filtered through Whatman No.42 filter paper. PNP released was measured spectrophotometrically at 420 nm. Standard set was run using different aliquots of working solution of 100 ppm p-nitrophenol mixed with $CaCl_2$, NaOH and followed by appropriate dilution with distilled water and the absorbance was measured at 420 nm. Standard graph was prepared by plotting the concentration of aliquots against their respective absorbance at 420 nm and the results of test solutions were plotted on it and the concentration of p-nitrophenol released was calculated accordingly. Unit activity expressed in terms of 1μg p-nitrophenol produced per mL per minute.

Urease activity of soil was measured by urea remaining method (Tabatabai, 1994). Soil samples were mixed with toluene and urea solutions and stoppered properly. Then all the samples were allowed to incubate at 37^0C for 5 hrs. After incubation, KCl-PMA was added and put them in shaking condition for 1 hr. after that, the samples were filtered through watman paper 42 and 2 mL of filtrate was taken in volumetric flasks for each treatment. Again the KCl-PMA solution was added to make up the final volume upto 10 mL and mixed with diacetyl monoxime which was used as color reagent and placed in boiling water bath for 30 minutes after which the samples were allowed to cool and then diluted with distilled water. Absorbance of red color developed in all samples was measured at 527 nm using spectrophotometer (Systronics 168). Here also standard graph was plotted using standard urea solution. Different aliquots of 250 µg/mL urea solution were mixed with KCL-PMA by making final volume upto 10 mL and the absorbance was measured at 527 nm.

3.9.7 Microbial parameters of soils:

To check the effect of different treatments on microbial properties of soil total viable count and actinomycetes count of soil was enumerated by Standard Plate Count (SPC) technique. For total viable count Nutrient agar medium was used whereas, for enumeration of Actinomycetes of soil Glucose Aspargine Agar (GAA) was used. For this, 1g soil from each pot at 40 and 65 DAS was added into sterile distilled water tubes and serial dilutions from all the soil samples were prepared ranged between 10^{-1} and 10^{-3} in sterile distilled water. From these dilutions 1 mL suspension was inoculated in melted media (nutrient agar for total viable count and GAA for actinomcetes) tubes and poured into sterile media (nutrient agar for total viable count and GAA for actinomcetes) plates followed by incubation at 37^0C for 24-48 hours. After incubation colonies were counted and enumerated as colony forming unit (CFU/g of soil) for tand al viable count and actinomycetes.

Diversity of fungi was also checked for all the soil samples. For this purpose, 1 g of soil samples were added to the sterile distilled water and allowed to settle down. Serial dilutions from 10^{-1} to 10^{-4} were prepared for each soil sample in sterile distilled water tubes. 0.1 mL from each soil dilutions were inoculated on Rose Bengal Agar medium

using spread plate technique and incubated at 37^0C for 3-7 days. After incubation, microscopic observation of all the different fungal colonies was performed to identify various fungi present in respective soil samples.

3.10 Degradation of monocrotophos in different types of soil:

3.10.1Soil analysis:

Soil is classified in different orders and each soil order has different physico-chemical properties. Purpose of this study was to evaluate the ability of *B. subtilis* KPA-1 to degrade the pesticide present in soils of different orders and to find out the effect of soil properties on the degradation rate of pesticide by potent bacterial culture. Thus, different soils representing seven orders alfinsol, vertisol, ultisol (humult), ultisol (ustult), aridisol, mollisol, inceptisol were collected from 0-25 cm depth from various regions and analyzed for varoius physico-chemical parameters including pH using pH-meter; total organic carbon by titrimetric method (Walkley and Black, 1934); available nitrogen using alkaline potassium permanganate method (Subbiah and Asija, 1956), potassium were measured using ammonium acetate extraction method and determined by flame photometer (Hanway and Hiedal, 1952; Richards, 1954), phosphorus by Olsen method (Olsen et al., 1954), moisture content by Gravimetric method, water holding capacity (Keen and Racozkowski 1921) and texture using hydrometer method (Bouyoucos, 1962). Heavy metals present in all soils were estimated by AAS using DTPA extraction method.

Apart from that, enzymatic study of all the soil samples representing different orders was done. Alkaline and acid phosphatase estimation was done as per Tabatabai and Bremmer, (1969) and Eivazi and Tabatabai, (1977). Urease activity was checked from the soil samples as per the method of urea remaining (Tabatabai, 1994). The number of soil microorganisms was enumerated by Most Probable Number (MPN) technique using nutrient agar medium for total viable count and Glucose Aspargine Agar (GAA) medium for Actinomycetes.

3.10.2 MCP degradation by B. subtilis KPA-1 in MCP contaminated soils:

Soil samples of all the seven soil orders were divided in three sets. In all the three sets i.e. T1, T2 and T3, 25g of each soil was taken in 250 mL conical flask and mixed with 1000 ppm MCP solution. In treatment T1, soils were kept unsterile. In treatments T2 and

T3, the soils were autoclaved before pesticide application by fractional sterilization method. In T3 treatment, soils were inoculated with potent culture of *B. subtilis* KPA-1 after sterilization. In other treatments, soils were kept uninoculated. All the sets were incubated for 144 hours in static condition at 35^0C temperature. After incubation, residual MCP was extracted stepwise by 30, 20 and 10 mL chloroform. After extraction, chloroform fraction was pooled out using separating funnel as stated earlier and evaporated at room temperature. After evaporation, residues were re-dissolved in minimum volume of ethyl acetate and residual MCP was then quantified by HPLC analysis as discussed earlier. The sterile, uninoculated soil samples were served as control. Nonsterile soil samples which kept uninoculated, were having indigenous microflora. Thus the ability of the potent culture to degrade the pesticide compared to that of the indigenous soil microflora was also checked.

3.11 Esterase and Phosphatase production by *Bacillus subtilis* KPA-1:

3.11.1 Enzyme extraction:

Enzymes produced by *B. subtilis* KPA-1 are responsible for biodegradation of monocrotophos which is an organophosphorus pesticide and phosphatase and esterase could be the responsible enzymes. To check the production of phosphatase and esterase by potent bacteria which are among the most important enzymes for organophosphorus pesticide degradation, conical flasks containing 20 mL LB (Luria Bertani) broth (g/l, trypton: 10, NaCl: 10, yeast extract: 5, pH:7) were inoculated with 1 mL inoculum of potent culture having inoculum density of 1 at 600 nm optical density and incubated at 37^0C for 24 hr at 150 rpm. After incubation, broth was centrifuged at 10,000 rpm, at 4^0C for 15 minutes and the cell pellets were collected and suspended in sterile distilled water. Then three sets of mineral salt medium amended with 1000 ppm MCP were inoculated with 1 mL potent culture having $O.D_{600}$ = 1and incubated as per optimized conditions. After incubation, the culture broth was centrifugaed at 10,000 rpm at 4^0C for 15 minutes. Supernatant of one set was used to estimate extracellular enzymes production whereas other sets were used to estimate production of intracellular and membrane-bound enzymes. For extracellular enzyme estimation the supernatant obtained was used as such whereas for intracellular and membrane-bound enzymes assay the pellets were used. For

intracellular and membrane-bound enzymes assay the pellets were dissolved in 50 mM, citrate phosphate buffer having 8 pH. Now the cells were disrupted by sonication with cooling on ice at 70% amplitude in four 30 sec periods, separated by 30 seconds cooling periods (SONICS Vibracell). After sonication, the sample was taken out and used as source of membrane bound as well as intracellular enzymes. The sonicated broth was centrifuged at 10,000 rpm for 15 minutes at 4^0C. After centrifugation, the supernatant was used as intracellular enzyme preparation and the pellets were dissolved in fresh sterile MSM medium broth and this fraction was used as membrane bound enzyme preparation. Phosphatase enzyme activities were determined by King and Armstrong method (King et al., 1951) and esterase assay was performed by Lee et al., (1999) from all the three preparations.

3.11.1.1 Phosphatase assay:

For estimation of phosphatase enzyme by King and Armstrong method, test sample was prepared my mixing buffer (0.2 M sodium acetate-acetic acid buffer, pH 4.9 for acid phosphatase and 0.1 M sodium carbonate- bicarbonate buffer, pH 10 for alkaline phosphatase) with disodium phenyl phosphate which was used as substrate and warmed at 37^oC followed by addition of enzyme source/extract and incubated at 37^oC for 15 minutes. Then after addition of TCA (Tri Chloro Acetic acid) the solution was filtered and used for estimation. Likewise, control was prepared by mixing water, instead of buffer and substrate, with enzyme source and TCA. While, blank was prepared using buffer, substrate and TCA without enzyme source. For enzyme assay, 2 mL of each test, control and blank were diluted with distilled water and treated with ammonium molybdate solution and after well mixing, stannous chloride was added as reducing agent and allowed to incubate for 15 minutes at 37^0C in incubator. Standard set was also run using KH_2PO_4 solution having 0.004 mg P/mL concentration. Different aliquots of standard were taken and rest of the assay was performed as per test samples. Here, blank was prepared using water, TCA, ammonium molybdate and stannous chloride solution. Color developed after incubation was measured spectrophotometrically at 660 nm and the unit activity was calculated by following formula,

Phosphatase activity U /mL/minute =

$$\frac{\text{Reading of test} - (\text{reading of control} + \text{reading of blank}) \times 0.004 \times 3}{\text{Reading of standard at 1 mL aliquote} \times 0.04 \times 15}$$

Here, concentration of Phosphorus in 1 mL standard solution is 0.004 mg and 2 mL of each test and control represents 0.04 mL enzyme. Unit phosphatase activity was expressed in terms of the enzyme which librates 1 mg phenol from p-nitrophenyl phosphate and it can equally well be defined as that which liberates 1/3 mg phoshorus thus it is necessary to multiply the activity with three. So, the unit phosphatase activity in present study has been expressed as enzyme that librates 1 mg phosphorus /mL/minute.

3.11.1.2 Esterase assay:

Esterase activity for all the sets was estimated by using p-nitrophenyl butyrate as substrate. Esterase activity was measured by mixing enzyme source with substrate-buffer solution which was prepared by acetone having p-nitrophenyl butyrate, ethanol and 50 mM citrate phosphate buffer (pH 8) followed by incubation at 50^0C for 15 minutes. Absorbance of the color developed was measured spectrophotometrically at 405 nm. One unit of esterase was defined as amount of enzyme releasing 1 µmol p-nitrophenol per mL per minute under assay condition. Standard was run using p-nitrophenol and the graph was prepared by plotting standard aliquots against respective absorbance at 405 nm. Regression equation was obtained from the standard plot and the esterase activity for test was determined by plotting absorbance of test samples in equation. Whole experiment was performed in triplicates.

3.11.2 Optimization of esterase production by OFAT approach:

After confirmation of esterase production by *B. subtilis* KPA-1, optimization of some important parameters like, temperature, pH, incubation time, inoculum size and age, carbon source and nitrogen source was carried out to enhance the esterase production.

3.11.2.1 Effect of incubation temperature:

To check the effect of temperature on esterase production by *B. subtilis* KPA-1, 1 mL inoculum of OD_{600} = 1 was inoculated in different 250 mL conical flasks containing 100

mL MSM broth supplemented with 1000 ppm MCP and incubated at different temperature ranges from 20 to 60^0C for 144 hours. After incubation, medium broth from all the flasks were centrifuged at 10,000 rpm at 4^oC for 15 minutes and the culture broth from all the flasks were used for esterase estimated by the method described above.

3.11.2.2 Effect of pH:

Optimization of pH was carried out preparing different 250 mL capacity flasks containing 100 mL MSM having 1000 ppm MCP. pH of medium in different flasks were adjusted in the range of 5 to 10 pH using 0.1 N HCl and NaOH and inoculated with 1 mL inoculum of potent bacterial culture. All the flasks were incubated at 37^0C for 144 hrs at 150 rpm. After incubation, culture broth from all the sets were used to obtain membrane bound enzyme fractions and used for esterase estimation.

3.11.2.3 Effect of incubation time:

Incubation time is one of the very important parameter for enzyme production by bacteria. Thus, most favorable incubation time was determined by inoculating different 250 mL capacity conical flasks containing 100 mL MSM medium having 1000 ppm MCP with 1 mL inoculum and each flask was incubated for different incubation period starting from 24 to 144 hrs. After incubation, culture broth from all the sets were taken out and esterase activity was determined for all the samples by standard method as above.

3.11. 2.4 Effect of inoculum age:

To identify the most favorable age of inoculum, 1 mL inoculum of 24 to 120 hr old culture having absorbance of OD_{600} = 1 was inoculated in different 250 mL capacity conical flasks flasks containing 100 mL MSM broth with addition of 1000 ppm MCP and the flasks were incubated for 144 hrs at 37^oC, 150 rpm. Supernatant from all the culture broths were obtained by centrifugation under conditions stated above after completion of incubation period and membrane bound enzyme preparations were prepared and were used to check the esterase production by bacteria.

3.11.2.5 Effect of inoculum size:

Inoculum volume of bacterial culture is crucial factor for bacterial production of enzyme. Thus inoculum size was also optimized for maximum esterase production and for that, 1 to 5 mL inoculum having density of OD_{600} = 1 were inoculated in different flasks having 100 mL MSM supplemented with 1000 ppm MCP and incubated in shaking condition at 150 rpm at 37^{o}C for 144 hrs. After incubation of all the sets, esterase estimation of membrane bound enzyme fractions collected from all the culture broth were performed by methods explained above.\\

3.11.2.6 Effect of carbon source:

Influence of additional carbon source on esterase production was also analyzed by adding 1% glucose, fructose, sucrose, cellobiose, cellulose, and maltose into various flasks of 100 mL MSM broth having 1000 ppm MCP and autoclaved. After sterilization, all the flasks were inoculated with the 1 mL potent culture followed by incubation at 37^{o}C, 150 rpm for 144 hrs. After incubation, broths from all the sets were taken out and membrane bound enxyme fractions were collected and used for esterase activity estimation by the method described earlier.

3.11.2.7 Effect of nitrogen source:

Nitrogen source affect the cell biomass and ultimately enzyme production by bacterial culture. Thus most suitable nitrogen source was optimized to achieve highest esterse production. Thus, different inorganic as well as organic nitrogen sources including ammonium sulfate, peptone, urea, yeast extract and meat extract were added to different sets of 100 mL MSM broth containing 1000 ppm MCP at 1% concentration. After that, all the flasks were autoclaved followed by inoculation with 1 mL inoculum of potent culture and incubated at 37^{o}C and 150 rpm for 144 hrs. Esterase production in presence of nitrogen source was determined by using the membrane bound enzyme preparation obtained from all the culture broth.

3.11.2.8 Effect of MCP concentration:

Effect of monocrotophos concentration on esterase production by *B. subtilis* KPA-1 was checked by adding different MCP into the 100 mL MSM medium at different concentrations viz. 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm followed by inoculation of the 1 mL inoculum of potent culture and incubated at 37^{o}C and 150 rpm for 144 hrs. After incubation the cell free supernatants were collected and used for esterase estimation.

During optimization of one parameter other cultural parameters were kept stable i.e. the incubation was done at 37^{o}C, 150 rpm for 144 hours and the pH of the medium was kept at 7 pH.

3.11.3 Optimization of alkaline phosphatase by OFAT approach:

Various parameters affect the microbial enzymes which need to be optimized to achieve maximum enzyme production. Thus, several important parameters like, incubation temperature, medium pH, incubation time, inoculum size, inoculum age, carbon source and nitrogen source were optimized to enhance the production of alkaline phosphatase by *B. subtilis* KPA-1.

3.11.3.1 Effect of incubation temperature:

To check the effect of incubation temperature on alkaline phosphatase production, 250 mL conical flasks were prepared containing 100 mL MSM broth which was having 1000 ppm MCP. All the flasks were sterilized by autoclaving followed by inoculation with 1 mL potent bacterial culture. After inoculation, each flask was incubated at various temperature starting from 20, 30, 40, 50 to 60^{0}C under shaking condition at 37^{0}C for 144 hrs.

3.11.3.2 Effect of medium pH:

Effect of initial pH of the medium on production of alkaline phosphatase was studied in the range of 5 to 11. pH of the 100 mL MSM broth having 1000 ppm MCP was adjusted by 0.1 N HCl or NaOH. Medium in all the flasks were inoculated with 1 mL

active culture of potent bacterium and incubated in shaking incubator at 150 rpm, 37^0C for 144 hrs.

3.11.3.3 Effect of incubation time:

To determine the favorable incubation time for alkaline phosphatase production, 1 mL potent culture having density of OD_{600} = 1 was inoculated in different sets of 100 mLMSM broth provided with 1000 ppm MCP. The flasks were then incubated in shaking condition at 150 rpm, 37^0C temperature. Each flask was then withdrawn at different time period with regular interval of 24 hrs starting from 24 to 144 hrs.

3.11.3.4 Effect of inoculum age:

Age of the inoculum used to inoculate the 100 mL MSM broth having 1000 ppm MCP was selected in the range of 24, 48, 72, 96 and 120 hrs for optimization study. For that, bacterial culture was incubated for different time periods separately i.e. 24 to 120 hrs. 1 mL of such inoculum having different age was then inoculated in different flasks containing medium and incubated at 150 rpm, 37^0C till 144 hrs.

3.11.3.5 Effect of inoculum size:

To enhance the alkaline phosphatase production by bacteria, inoculum concentration of bacterium was also investigated. For that, 250 mL capacity conical flasks were prepared having 100 mL MSM broth supplemented with 1000 ppm MCP and sterilized before inoculation. After that, different volumes of inoculum viz. 1, 2, 3, 4 and 5 mL were inoculated in different flasks followed by incubation at 37^0C, on a rotary shaker 150 rpm for 144 hrs.

3.11.3.6 Effect of additional carbon source:

Effect of various additional carbon sources on alkaline production was also evaluated. For that purpose, 100 mL MSM medium containing 1000 ppm MCP was supplemented with 1% concentration of glucose, mannose, fructose, sucrose, cellulose and cellobiose in different conical flasks. After that, each flask was inoculated with 1 mL potent inoculum and incubated at 150 rpm, 37^0C for 144 hrs.

3.11.3.7 Effect of nitrogen source:

Another important medium component for enzyme production by bacteria is nitrogen source which was also optimized by adding different nitrogen sources to the medium. Ammonium sulfate, peptone, yeast extract, meat extract and urea were added to the 100 mL MSM broth having 1000 ppm MCP medium in different conical flasks at 1.0% concentration. All the flasks were inoculated 1 mL potent culture having absorbance OD_{600} = 1 and incubated till 144 hrs under shaking condition (150 rpm) at 37^0C.

3.11.2.8 Effect of MCP concentration:

MCP concentration added into the medium was optimized to achieve maximum alkaline phosphatase production from *B. subtilis* KPA-1. For this purpose, MCP in the range of 100 to 1000 ppm were added to different flasks of 100 mL MSM medium. After that, 1 mL inoculum of *B. subtilis* KPA-1 was inoculated in all the flasks followed by incubation at 37^0C for 144 hrs.

All the other parameters were kept stable except one which has to be optimized during study. After incubation, medium broth of each flask was taken out and centrifuged at 15,000 rpm for 15 minutes at 4^0C for all the parameters. After centrifugation, the supernatants were discarded and cell pellets were dissolved in 50 mM, citrate-phosphate buffer (8 pH) and sonicated as per earlier stated conditions. After sonication, the enzyme extracts were again centrifuged at 15,000 rpm for 15 minutes at 4^0C and the supernatants were used to estimate the alkaline phosphatase production in each sample by the method explained earlier.

After observing the results of optimization study, it was very much clear that, carbon source and nitrogen source are the most effective factors for alkaline phosphatase production by *B. subtilis* KPA-1.

3.11.4 Optimization of esterase and alkaline phosphatase production by statistical approach:

From the optimization study by OFAT approach, it was revealed that, carbon source and nitrogen source are the most significant factors that affect both the esterase and alkaline phosphatase production by *B. subtilis* KPA-1. Moreover, this study is regarding

the potential of bacteria to grow in presence of pesticide and to degrade it. Apart from that, bacterial strain is also capable of utilizing the pesticide as carbon and energy source. Thus, concentration of three most important factors i.e. carbon source, nitrogen source and pesticide were optimized to enhance the production of alkaline phosphatase and esterase by *B. subtilis* KPA-1. The concentration of other media constituents was kept constant throughout the whole study. The effect of three macronutrients glucose (carbon source), ammonium sulfate (nitrogen source) and monocrotophos (pesticide) on the production of both the enzymes by *B. subtilis* KPA-1 was evaluated at five different coded levels (-1.682, -1, 0, +1,+1.682) and 2^3 factorial Central Composite Experimental Design with six start points and six replicates at the central point, resulting in 20 experiments was generated by Design Expert, Version 7.0, Stat-Ease Inc., Minneapolis (MN) statistical software. The experimental design and levels of factors used for experimental design are given in **Table 4.25** and **4.26**. The coded variables are calculated according to the equation 1. As per the results obtained from experimental design, polynomial relationship between independent variables was obtained (equation 2) and the response was used to fit the second-order polynomial equation (eqution 3).

Equation 1:

$$X_i = \frac{(X_i - X_i^x)}{\Delta X_i}, \qquad \text{i} = 1,2,3\ldots\ldots, \text{j}$$

From the results obtained after performing experiments, approximate polynomial relationship for dependent variables for esterase production was obtained and the relationship of variables and response was calculated as per following second order polynomial equation:

Equation 2:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \epsilon$$

Where, Y = predicted response

ϵ = random error

k = 3 (as there are three independent variables)

Thus, the mathematical relationship between three variables and the response from above equation becomes,

Equation 3:

$$Y = \beta_0 + B_1A + B_2B + B_3C + B_1A^2 + B_2B^2 + B_3C^2 + B_{12}AB + B_{23}BC + B_{13}AC$$

Where, Y= predicated response

B_0 = offset term

B_1, B_2, B_3 = linear coefficient

B_{12}, B_{23}, B_{13} = cross product coefficient

A, B, C = independent variables

3.11.5 Purification of esterase and alkaline phosphatase enzyme:

After optimized the condition for maximum enzyme production, both the enzymes were purified further. To purify both the enzymes, two sets of 100 mL MSM medium having 1000 ppm MCP was prepared and incubated according to the respective optimized conditions and the enzyme preparations were obtained as described earlier. Among extracellular, intracellular and membrane-bound enzymes preparations the maximum alkaline phosphatase and esterase activity giving preparations were further studied for purification. At each successive purification step the samples were analyzed to assess the esterase activity, protein content, specific activity and fold purification.

3.11.5.1 Ammonium sulfate precipitation:

The very first step for partially purification of enzyme was ammonium sulfate precipitation which was done by addition of small increments of ammonium sulfate into medium broth containing enzyme with constant stirring by means of magnetic stirrer and the temperature was maintained at 4^0C using ice and ice-pack to obtain four fractions i.e. 5%, 10%, 20% and 30% of ammonium sulfate. When all the amount of ammonium sulfate got dissolved, the mixture was allowed to stand for overnight at cooling temperature after each fractionation. Then the supernatant and pellets were separated by centrifugation at 10,000 rpm for 15 minutes at 4^0C and used for both enzymes and protein

estimation. Esterase estimation was done as described earlier using p-nitrophenyl butyrate as substrate and mixing the enzyme source with substrate-buffer solution (prepared by acetone having p-nitrophenyl butyrate, ethanol and 50 mM citrate phosphate buffer pH 8) followed by incubation at 50^0C for 15 minutes and recording absorbance of the color developed by spectrophotometer at 405 nm. Whereas, alkaline phosphatase estimation was done by King and Armstrong method as described above using p-nitrophenyl phosphate as substrate. Protein content was estimated by Folin-Lawry method (Lowry et al., 1951). For estimation of protein content, enzyme preparation was diluted by distilled water. After that, a mixture of sodium carbonate prepared in NaOH and sodium potassium tartrate prepared in $CuSO_4.7H_2O$ was added to it followed by addition of Folin cio-calteu reagent and incubated in dark for 30 minutes. Similarly, standard was prepared by using bovine serum albumin at concentration of 200 µg/ mL and run as per test solution. After development of blue color, color intensity was measured spectrophotometrically at 650 nm.

3.11.5.2 Gel filtration chromatography:

Gel filtration chromatography was also used for further purification of enzymes. here, Sephadex G-200 was used as packing material and the gel was prepare by dissolving 2 g sephadex G-200 in 30 mL of citrate-phpsphate buffer, 50 mM, 8 pH. The prepared packing material was then loaded in glass column. For purification of enzyme, 200 µL of concentrated enzyme dissolved in citrate-phosphate buffer (pH 8) and diluted with 2 mL of the same buffer and 500 µL of such diluted enzyme was then loaded on to a sephdex G-200 column (2 cm × 30 cm). The column was washed with milique water and pre-equilibrated with 50 mM citrate-phosphate buffer (pH 8). 50 fractions of 2 mL were collected and esterase as well as alkaline phosphatase activities were estimated from each fractions by above stated method and the protein content was also measured at 280 nm by UV spectrophotometer (Analytik jena, specord 200+).

3.11.6 SDS-PAGE gel electrophoresis:

SDS-PAGE electrophoresis of partially purified enzyme was carried out using a vertical gel apparatus (Biorad powerpac Basic with Mini PROTEAN tetra system).

Native and SDS-PAGE both were performed with 10% resolving and 4% stacking gel. Native or non denaturing PAGE electrophoresis was performed without using SDS. To perform electrophoresis, plates and spacers were cleaned with alcohol and spacers were placed between glass plates with the help of clamps. Plates were sealed with agar from below. After that, 10% resolving gel prepared as described below was poured into the space between plates by pipette and allowed to solidify.

- Casting of resolving or separating gel:
  15 mL of resolving gel was prepared by mixing following:

Deionized water	- 5.9 mL
Acrylamide	- 5 mL
Tris-buffer (pH 8.8)	- 3.8 mL
SDS (10%)	- 0.15 mL
Ammonium persulfate	- 200 µL
TEMED	- 20 µL

The mixture was mixed properly by swirling the flask and then poured in the gel plate and allowed polymerized.

- Casting of stacking gel:
  3 mL of stacking gel was prepared by mixing the following:

Deionized water	- 2.1 mL
Acrylamide	- 0.5 mL
Tris-buffer (pH 6.8)	- 0.38 mL
SDS (10%)	- 0.03 mL
Ammonium persulfate	- 100 µL
TEMED	- 10 µL

Appropriate comb was inserted between gel plates. The mixture of stacking gal was mixed properly by swirling the flask and then poured over resolving gel. After its polymerization, the comb was removed and the wells were washed with buffer to remove the remaining unpolymerized acrylic acid. For sample preparation, enzyme fractions

having maximum protein content as well as enzyme activities were used. 15 µL of such samples was mixed with 10 µL of 1X SDS loading dye. Protein marker was also prepared by same way. A medium range protein standard [Geni (merck), catalogue no. 623110275001730] was used as molecular weight marker which contained phosphorylase b (97.4 kDa), bovine serum albumin (66.0 kDa), ovalalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). The gel apparatus was then fitted in chamber and filled with electrophoresis buffer. Appropriate amount of samples were then loaded in wells. Power supply was then turned on and gel was allowed to run till the dye reached at bottom of the gel. The gel was then stained by silver staining method (Simon, 2004). After removing the gel from the apparatus, stacking gal was removed and the remaining gel was washed stepwise with methanol, milique water, sodium thiosulfate (0.02%). After that, it was kept in dark with 0.2 % $AgNO_3$ and it was followed by washing with milique water. After that, developing agent containing Na_2CO_3, sodium thiosulfate and formaldehyde was poured on the gel and swirled till the bands appeared. 10% glacial acetic acid or 1.4% Na_2EDTA was used as stop solution. The molecular weight of protein was then determined by comparing the bands with that of protein marker.

3.11.7 Characterization of esterase enzyme:

The partially purified esterase enzyme was used for its characterization. During characterization, effect of pH, temperature, substrate concentration, enzyme concentration, incubation period, metal ions, organic solvents, metal inhibitors and other chemical agents was evaluated by using p-nitro phenyl butyrate as substrate.

Effect of temperature on enzyme was determined by incubating the tubes containing enzyme reaction mixture (enzyme and substrate buffer solution of pH 8) at wide range of temperature (30, 40, 50, 60, 70 and 80^0C) for 15 minutes. After incubation the absorbance of the color developed was measured by spectrophotometer at 405 nm and the relative activity of enzyme was measured by taking the activity of control sample as 100%. Likewise for determining pH optima for enzyme activity the enzyme reaction mixture (containing enzyme and substrate buffer solution of pH 4, 5, 6, 7, 8 and 9) was incubated at above optimizes temperature for 15 minutes followed by reading absorbance

at 405 nm. The buffer solutions used for this pH optima are- 50mM of sodium-acetate buffer of 4 and 5 pH, phosphate buffer of 6 and 7 pH, citrate buffer of 8 pH and glycine-NaOH buffer of 9 pH. To determine the effect of substrate concentration on esterase enzyme activity the enzyme was mixed with substrate-buffer solution of above optimized pH. Here different concentrations of substrate (p-nitro phenyl butyrate) used are- 5, 10, 15, 20, 25 and 30 mM. After mixing the mixture was incubated at above optimized temperature for 15 min. followed by OD measurement. Effect of enzyme concentration on activity was measured by using different aliquots of enzyme viz. 0.15, 0.30, 0.45, 0.60 and 0.75 mL in enzyme reaction mixture and the assay was carried out under above optimized conditions. Incubation period optima was evaluated by incubating the enzyme reaction mixture for 5, 15, 25, 35, 45, 55 and 65 minutes at optimized temperature and the relative activity was measured. During characterization of one factor other parameters were kept constant as standard method.

To evaluate the effect of different metal ions, enzyme was pre-incubated for 30 minutes at 37^0C with salts of metals like $CaCl_2$, $MgCl_2$, $CuCl_2$, $FeCl_3$, $NiSO_4$, $MnCl_2$, $ZnCl_2$ at 1 mM concentration before estimation followed by estimation of esterase activity as per optimized conditions. Apart from that, influence of various chemicals like EDTA (Ethylene Diamine Tetra Aceticacid), DMSO (Dimethyl sulfoxide), PMSF (Phenylmethanesulfonylfluride), triton-X100, β-mercaptoethanol and SDS (Sodium dodecyl sulfate) on esterase activity was checked by incubating them for 30 minutes at 37^0C with enzyme before performing enzyme assay. Organic solvents viz. methanol, acetonitrile, ethanol, 1-butanol and acetone was also added in enzyme preparation to see the response of enzyme towards them at 1 M concentration and incubated for 30 minutes at 37^0C. Then the enzyme assay was performed as per method discussed above using p-nitro phenyl butyrate as substrate incubated at optimized temperature for optimized time. The relative or residual activity (%) was determined under above optimized conditions in absence of any additive which was considered as 100% in all cases.

3.11.8 Substrate specificity of esterase enzyme:

Substrate specificity of partially purifird esterase enzyme produced by *B. subtilis* KPA-1 was checked using different substrtaes. 1 mM solutions of p-Nitrophenyl acetate,

butyrate, caproate, caprylate, caparate, laurate, myristate and palmitate were prepared in citrate-phosphate buffer (50 mM, pH:8). Esterase estimation was done in different sets using different substrates under similar conditions.

3.11.9 Characterization of alkaline phosphatase enzyme:

The partially purified alkaline phosphatase enzyme was characterized to find out the optimum pH of medium, incubation temperature, substrate concentration, enzyme concentration, incubation period and effect of metal ions, organic solvents, metal inhibitors and other chemical agents on activity of phosphatase. The substrate used for this study was was p-nitro phenyl phosphate. For characterization one parameter was optimized at a time keeping other parameters constant. For enzyme estimation test, blank and control samples were prepared. Test contains enzyme, buffer, TCA (Tri Chloro Acetic acid) and substrate whereas blank contains buffer, substrate and TCA, and control has water, enzyme and TCA.

To determine the effect of incubated temperature on phosphatase enzyme, standard alkaline phosphatase assay was performed in 15 test tubes. Five incubation temperature were selected viz. 15, 25, 35, 45 and 55^0C and each incubation temperature comprises three test tubes each for blank, test and control. The blank, test and control test tube contains their respective solutions followed by addition of ammonium molybdate and stannous chloride. Now these 15 test tubes were incubated at selected temperatures such that each temperature should include one blank, one test and one control tube. After incubation the relative activity of enzyme was measured in all the 15 tubes. Response of phosphatase enzyme towards different pH was evaluated by performing the enzyme assay by incubating the enzyme reaction mixture with various buffers including 50 mM of sodium-acetate buffer of 5 pH, phosphate buffer of 6 and 7 pH, citrate buffer of 8 pH and glycine-NaOH buffer of 9 pH, carbonate-bicarbonate buffer of 10 and 11 pH. Then enzyme assay was performed as per standard method and relative activity was measured. Likewise, effect of substrate concentration was evaluated by performing enzyme assay using different concentrations of p-nitro phenylphosphate viz. 2, 4, 6, 8, 10, 12 and 14 M and relative activity was measured. Effect of enzyme concentration on activity was measured by using 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mL in enzyme reaction mixture and

the assay was carried out as above stated method. Incubation period optima was evaluated by incubating enzyme reaction mixture for 5, 15, 25, 35, 45, 55 and 65 minutes and the relative activity was measured

To evaluate the effect of different metal ions, 1mM solutions of $CaCl_2$, $MgCl_2$, $CuCl_2$, $FeCl_3$, $NiSO_4$, $MnCl_2$, $ZnCl_2$ were prepared in distilled water and now the enzyme was preincubated in these solutions for 30 minutes at 37^0C before enzyme assay. Test, control and blank were prepared as stated above and phosphatase enzyme assay was performed using these solutions as enzyme source. To study the effect of various chemicals like EDTA, DMSO, PMSF, triton-X100, β-mercaptoethanol and SDS on phosphatase activity the test, blank and control prepared above were subjected to color development for measurement of phosphatase activity taking care that in test and control the above preincubated enzyme with chemicals was used. Organic solvents viz. methanol, acetonitrile, ethanol, 1-butanol and acetone was also added to the at 1 M concentration to the enzyme and incubated for 30 minutes at 37^0C. Then the enzyme assay was performed as per standard method. The relative or residual activity (%) was determined under standard conditions. enzyme activity in absence of any additive was considered as 100% in all cases.

3.11.10 Enzyme kinetic study of esterase and alkaline phosphatase enzymes:

Enzyme kinetic study shows the rate of reaction catalyzed by enzyme and the effect of substrate concentration on the reaction. The Michealis Menten constant (km), maximum velocity (Vmax), turnover number (kcat) were calculated. Km is the concentration of substrate at which, the enzyme gives maximum velocity (Vmax). These kinetic parameters of partially purified esterase enzyme were studied by performing esterase assay using different concentration of p-nitrophenyl butyrate ranging from 5, 10, 15, 20, 25 and 30 mM concentration under optimum conditions. whereas, to study the kinetic parameters of alkaline phosphatase enzyme was fixed volume of alkaline phosphatase enzyme was incubated at different concentrations of substrate (p-nitrophenyl phosphate) in the range of 2, 4, 6, 8, 10, 12 and 14 M under optimized conditions. Lineweaver - Burk plot was prepared by plotting reciprocal of the enzyme activities (1/V0) against substrate concentration (1/S). Equation Y = mX + C was obtained from

this Line-Weaver Bulk plot from which values of C (slop) and m were obtained which were then fitted in Line-Weaver Bulk plot equation to know the Km and Vmax values. Km and Vmax were calculated by following equations:

Slop=Km/Vmax,

Where, Vmax = 1/C (slop)