

Optimization Study of Esterase Production by Monocrotophos Degrading Bacterium *Bacillus subtilis* KPA-1

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Production of esterase enzyme which is the key enzyme for organophosphorus pesticide degradation from *Bacillus subtilis* KPA-1 was confirmed in present study. Esterase production from *Bacillus subtilis* KPA-1 was observed when inoculated in mineral salt medium and localization of esterase production was also determined and the esterase enzyme was found to be membrane bound enzyme. Various process parameters were optimized by conventional method for maximum esterase production by *B. subtilis* KPA-1. Results indicate that pH 8, incubation temperature of 40°C, incubation time of 72 hr, 48 hr old inoculum with 3% inoculum size, glucose as carbon source and ammonium sulfate as nitrogen source and 800ppm monocrotophos concentration were found to be optimum values for highest esterase production.

Keywords: Pesticide degradation, fermentation conditions, esterase.

Esterases (EC 3.1.1.1) belong to hydrolases class of enzymes which catalyze the cleavage and formation of short chain fatty acid esters. They have broad range of substrate specificity, highly chemo-, region- and enantio-selectivity and non-aqueous catalytic activity¹. Esterase catalyzes reactions like interesterification, intraesterification and transesterification. Esterases (EC 3.1.1.1) are widely found in animals and plants but microorganisms are prominent sources for esterase production. All classes of microorganisms like bacteria, fungi, actinomycetes produces esterases- either constitutively or it is inducible². There are many bacterial strains which are reported as producers of esterases viz. *Bacillus licheniformis*³, *Bacillus megaterium*⁴, *Bacillus* sp⁵, *Sulfolobus tokodaii*⁶, *Streptococcus thermophilus*⁷ and *Pseudomonas* sp.⁸. Esterase-producing bacteria have been found in various habitats including soil contaminated with oil, dairy

waste, industrial wastes, oil seeds and decaying food, compost heaps, coal tips and hot springs⁹.¹⁰. Esterases have wide range of applications in various fields including ester synthesis, food and textile industries, paper industry, detergent, drugs, in manufacturing of liquid glue, cosmetics, meat tenderization, cheese production, growth promoters etc.^{11,12}. They are also useful to modify the chemical properties of triglycerides in oil and fat industry^{13, 14, 15} as well as biodiesel and biopolymer production^{16, 17, 18}. Bacterial strains including *Pseudomonas*, *Comamonas* and *Bacillus* are also used for plastic degradation². Apart from that, they are also used for detoxification of organophosphorus compounds found in insecticides in agricultural industries¹⁹. *Bacillus* sp. isolated from marine sludge than can produce esterases which are used in hydrolysis of esters found in pesticide²⁰. Esterase production by bacteria in synthetic medium is depends upon cultural conditions and medium components. There are many such factors which can stimulate or suppress the production of esterase like, pH, temperature, incubation time, amount and age of

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inoculum used for the production and type and concentration of carbon and nitrogen sources added in medium. Thus, optimization of these parameters is most important to enhance the production of esterase by bacteria which also affect the economy of fermentation process. Improvement of esterase production still depends on the optimization of culture conditions, including the composition of the culture medium such as carbon and nitrogen sources and other fermentation parameters such as dissolved oxygen, temperature and aeration rate ²¹. Present study includes the conventional method of optimization to optimize the important parameters for the production of esterase by *Bacillus subtilis* KPA-1 and to screen out the most effective parameters. Conventional methods for optimization of medium and fermentation conditions are time consuming and expensive as well as not so accurate. Thus, response surface methodology (RSM) has been used to evaluate the effect of interactions of crucial medium components to enhance the esterase production.

MATERIALS AND METHODS

Chemicals

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

Preparation of bacterial inoculum

Bacillus subtilis KPA-1 was used in present study for esterase production which was isolated from farm soil samples by enrichment culture technique. This bacterial culture was able to degrade 1000 ppm monocrotophos (MCP) which is an organophosphorus pesticide. Inoculum of *B. subtilis* KPA-1 was inoculated in 20 mL of LB broth medium (g/l, trypton: 10, NaCl: 10, yeast extract: 5, pH: 7) and incubated at 37°C for 24 h under shaking condition at 150 rpm. After incubation, the culture broth was centrifuged at 7000 rpm at 4°C for 15 minutes. After centrifugation, supernatant was discarded and the cell pellets were washed with sterile distilled water and the cell mass was suspended in sterile distilled water and the optical density was adjusted to 1 at 600 nm which was

used as bacterial inoculum to achieved the optical density 1 at 600 nm.

Enzyme production by *B. subtilis* KPA-1

Esterase production by *B. subtilis* KPA-1 was confirmed and simultaneously, location of esterase production was also determined by inoculating bacterial culture of *B. subtilis* KPA-1, into 100 mL of Mineral Salt Medium (MSM) (K_2HPO_4 : 1.5, KH_2PO_4 : 0.5, $MgSO_4$: 0.2, $(NH_4)_2SO_4$: 0.5, NaCl: 0.5, $FeSO_4$: 0.02, $CaCl_2$: 0.05, pH: 7) containing 1000 ppm MCP. 1% v/v of bacterial inoculum of *B. subtilis* KPA-1 was inoculated in medium and incubated at 37°C under shaking condition at 150 rpm for 144 hrs. Culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C followed by incubation and the supernatant was used to estimate the extracellular enzyme activity. While, the biomass was suspended in citrate-phosphate buffer (50 mM, pH 8) followed by ultrasonication with cooling on ice at 70% amplitude in four 30 sec periods, separated by 30 seconds cooling periods. After ultrasonication, the broth was centrifuged again at 10,000 rpm for 15 minutes at 4°C and the supernatant was used as intracellular enzyme source while the cell pellets were re-suspended in fresh, sterile MSM medium which was used as membrane-bound enzyme fraction.

Enzyme assay

Esterase assay for all the three enzyme fractions was performed as per method described by ²². The reaction mixture having 1.8 mL of substrate-buffer mixture containing 1 mL acetone, 4 mL ethanol and 0.0034 g p-nitrophenyl butyrate in 94 mL, 50 mM citrate phosphate buffer (pH 8) and 0.6 mL crude enzyme was incubated for 15 minutes at 60°C. After incubation, the color was developed and the intensity of color was measured at 405 nm. One unit of esterase was defined as amount of enzyme releasing 1 μ mol p-nitrophenol per minute under assay conditions. 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.

Optimization of production parameters by OFAT approach

Different process parameters like, temperature, pH, incubation time, inoculum age and size, carbon source, nitrogen source and

monocrotophos concentration were optimized to enhance the esterase production by *B. subtilis* KPA-1 by OFAT (One Factor at A Time) approach.

Effect of temperature on esterase production was determined by inoculating different sets of 250 mL flasks containing 100 mL MSM medium with 1% v/v bacterial inoculum followed by incubation at various temperature ranges from 20 to 60°C. After incubation the medium from each flask was centrifuged at 10,000 rpm, at 4°C for 15 minutes.

Medium of the pH was also optimized for maximum esterase production by *B. subtilis* KPA-1. Different 250 mL flasks containing 100 mL MSM medium were prepared and pH of the medium in each flask was adjusted in the range of 5 to 10 using 0.1 N HCl or NaOH followed by inoculation with 1% v/v bacterial culture. All the flasks were incubated for 144 hrs at 30°C, 150 rpm followed by centrifugation at 10,000 rpm, at 4°C for 15 minutes.

Incubation time provided for enzyme production is one of the most important factors which was optimized by inoculating 1% v/v bacterial culture to different flasks containing 100 mL MSM medium which were then incubated at 30°C under shaking condition for 144 h. Samples were withdrawn at regular interval of 24 hr and centrifuged as stated earlier.

Inoculum size and age of *B. subtilis* KPA-1 are crucial parameters for enzyme production. For optimization of inoculum size, 1 to 5% v/v inoculum were inoculated into the 250 mL flasks containing 100 mL MSM medium and incubated at 37°C for 144 hr at 150 rpm. While, 1% v/v inoculum of 24 to 144 hrs old *B. subtilis* KPA-1 culture was prepared for esterase production. These inoculums were inoculated into different flasks having 100 mL MSM medium and incubated for 144 hrs at 30°C, 150 rpm.

Effect of additional carbon sources was checked by adding 1% solutions of glucose, sucrose, maltose, cellulose, cellobiose to the different sets of 100 mL MSM medium and inoculated with 1% v/v culture of *B. subtilis* KPA-1 followed by incubation at 37°C, 150 rpm for 144 hr. After incubation, the medium from each set was centrifuged and supernatant was used for enzyme estimation.

Apart from, carbon source, nitrogen source is also an important factor that affect the

enzyme production by bacterium. Thus, 1% of ammonium sulfate, peptone, urea, meat extract and yeast extract were prepared and added to different flasks of 100 mL MSM medium inoculated with 1% v/v bacterial inoculum and incubated as stated earlier.

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°C and 150 rpm for 144 hrs.

In all the cases, culture broth obtained after incubation were centrifuged as described before and the biomass was suspended in citrate phosphate buffer (8 pH, 50 mM) followed by sonication. Sonicated broth was centrifuged and the cell mass was resuspended in citrate-phosphate buffer (8 pH, 50 mM) and used as membrane-bound enzyme preparation in each case. During optimization of one parameter other cultural parameters were kept stable i.e. the incubation was done at 37°C, 150 rpm for 144 hours and the pH of the medium was kept at 7 pH.

RESULTS

Enzyme production by *B. subtilis* KPA-1

Three enzyme preparations obtained for esterase estimation were examined for esterase assay and it was revealed that, the cell free extract of *B. subtilis* KPA-1 which was served as extracellular enzyme showed very less amount of esterase activity i.e. 0.11 U/mL/min. While, the supernatant obtained after centrifugation of sonicated cell mass showed only 0.02 U/mL/min esterase activity. Compared to both preparations, membrane-bound enzyme preparation gave highest esterase activity i.e. 0.40 U/mL/min unoptimized conditions which suggests that the location of enzyme may be in outer membrane of the bacterial cell (Table 1). Similarly, *Bacillus subtilis* RRL-1789 able to give 0.22 U/mg esterase activity and enzyme was found to be located in outer membrane of the cell. Membrane-bound esterase enzyme production by bacteria has been also reported by other researchers including ^{23, 24, 25, 26, 27}. Esterase is one of the important enzymes for microbial

degradation of organophosphorus pesticide. Esterase production by *Bacillus subtilis* KPA-1 was estimated using p-nitrophenyl butyrate as substrate. Role of esterase enzyme in monocrotophos has been also suggested²⁸. The production of esterase enzyme by monocrotophos degrading bacteria and its role in pesticide degradation was proved by the fact that, the

researchers observed methylamine as one of the metabolites during monocrotophos degradation which can be produced by hydrolytic action of esterase enzyme. Organophosphates compounds are composed of three phosphoester linkages and the phosphorus is also oxygen or sulfur by double bond thus, the breakdown of these linkages could be the way to degrade the organophosphorus compounds and the esterase could be responsible enzyme for it^{29,19}. Involvement of esterase enzyme in malathion degradation by *Bacillus thuringiensis* MOS-5 was also observed³⁰. Role of microbially produced esterase in degradation of organophosphorus pesticide was also described^{31,32}. Production of esterase enzyme by carbofuran and methomyl degrading bacteria³³. Similarly, esterase production by *Bacillus* strain C5 which was able to degrade methyl-parathion was also observed²⁰.

Table 1. Esterase activity for different preparations

Enzyme preparation	Esterase activity (U/mL/min)
Extracellular extract	0.11
Membrane bound preparation	0.40
Intracellular preparation	0.02

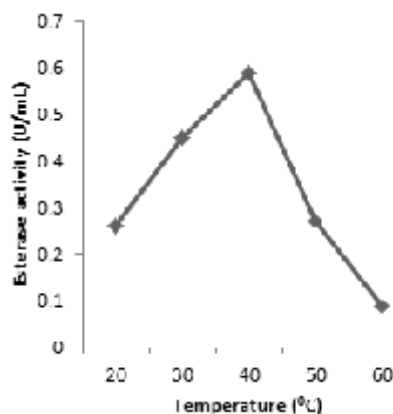


Fig. 1. Effect of temperature on esterase production

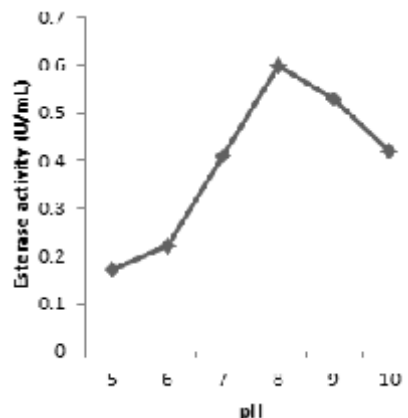


Fig. 2. Effect of pH on esterase production

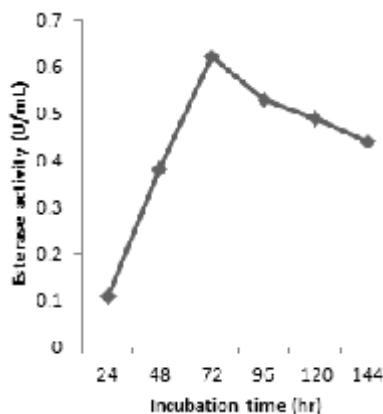


Fig. 3. Effect of incubation time on esterase production

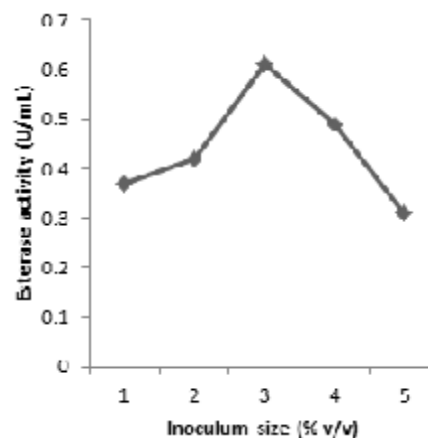


Fig. 4. Effect of inoculum size on esterase production

Optimization of cultural conditions for esterase production by *B. subtilis* KPA-1 by OFAT approach

Effect of temperature

Optimum temperature for esterase production by *B. subtilis* KPA-1 was checked in range of 20 to 60°C and maximum activity was observed at 40°C i.e. 0.59 U/mL (Fig 1). Increase or decrease in temperature showed reduction in enzyme activity. Similarly, it has been reported that, lipase production by *Bacillus coagulans* was found maximum at 40°C that decrease with increasing temperature³⁴; while, esterase production by *Lactobacillus brevis* NJ13 was

found highest at 50°C³⁵. It was also observed that, lipase production by *Bacillus* sp. was maximum at 40°C³⁶. Lipase production by *Achromobacter xylosoxidans* TS2MCN and *B. thuringiensis* TS11BP was also maximum at 45°C³⁷. Similarly, *Bacillus circulans* MAS2, isolated and also showed maximum esterase production at 50°C, they also found maximum bacterial growth at similar temperature and suggested correlation between bacterial growth and its enzyme production³⁸. While, maximum esterase production was recorded at 30°C by *Lactobacillus casei* CL96³⁹.

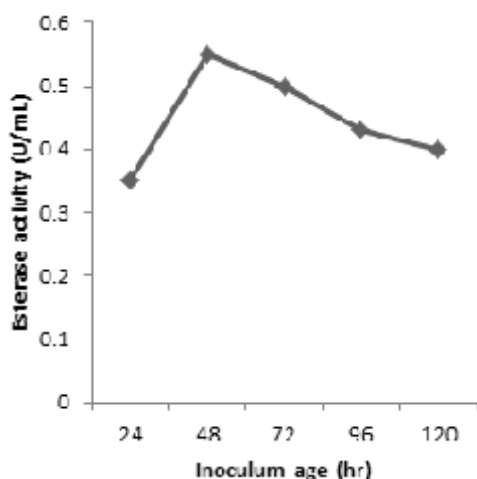


Fig. 5. Effect of inoculum age on esterase production

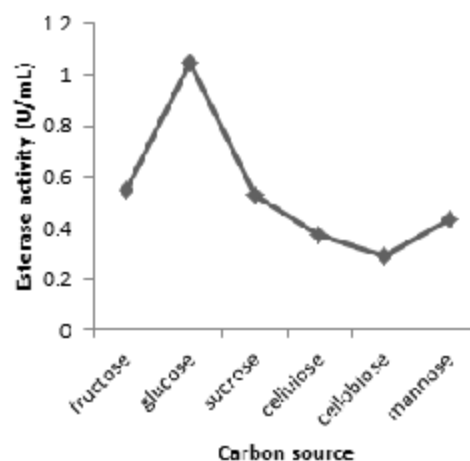


Fig. 6. Effect of carbon source on esterase production

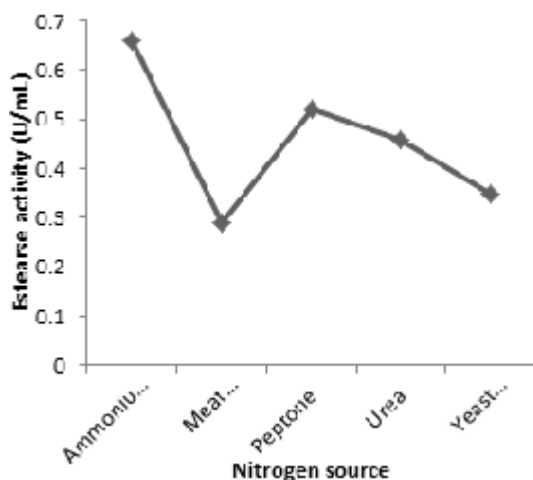


Fig. 7. Effect of nitrogen source on esterase production

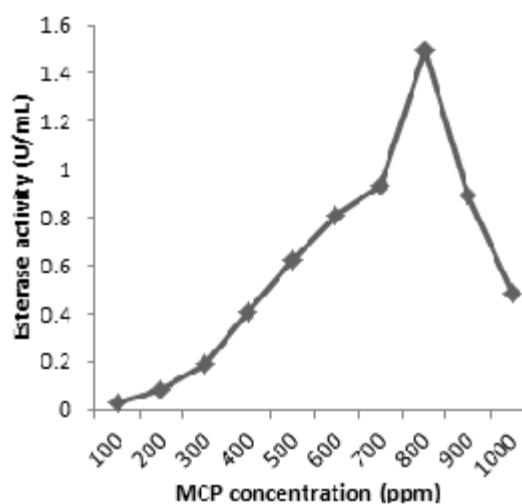


Fig. 8. Effect of monocrotophos concentration on esterase production

Effect of pH

Influence of pH of the medium on esterase production was analyzed in range of 5-10 pH. Highest esterase activity i.e. 0.60 U/mL was found when pH of the medium was adjusted at 8. Enzyme activity reduced gradually at pH higher than 8 but it reduced drastically at acidic pH which shows alkaline nature of the enzyme (Fig 2). According to the observations, little amount of elevation i.e. 29.41 % was obtained when pH changes from 5 to 6. Whereas, gradual and very sudden enhancement in esterase activity of 86.36 and 46.34 % was found when pH of the medium was adjusted to 7 and 8. Then after 11.47 and 20.75 % decrease in esterase activity was recorded with increment in pH upto 9 and 10. This result is in support with the findings that, *Bacillus circulans* produced esterase enzyme over wide range of pH from 7-9 but the production was found maximum at 9 pH⁴⁰. Likewise *B. coagulans* also produced maximum esterase enzyme at 8 pH³⁴. pH 8 was also found to be optimum for lipase production by *Bacillus* sp.³⁶ and *Achromobacter xylosoxidans* TS2MCN and *B. thuringensis* TS11BP³⁷.

Effect of incubation time

Production of enzyme by bacteria is also influenced by incubation time which was optimized in range of 24-144 hours and 72 hours of incubation period was found to be optimum at which the activity was 0.62 U/mL (Fig 3). Gradual and drastic increase in enzyme production was observed from 24 to 72 hr but decreased gradually after that. These results are supported in another study, in which maximum esterase enzyme production was obtained by *Vibrio fischeri* after 72 hrs of incubation period⁴¹. On the contrary, *Achromobacter xylosoxidans* TS2MCN and *B. thuringensis* TS11BP showed maximum lipase activity after 96 hr incubation³⁷.

Effect of inoculum size and age

Bacterial inoculum is one of the crucial factors for enzyme production. So, 1 to 5 % v/v bacterial inoculum were added to the medium for enzyme production and highest enzyme production i.e. 0.61 U/mL was observed when 3% v/v inoculum was added to the medium. Further increase or decrease in inoculum caused reduction in activity (Fig 4). Lipase production from *Serratia* sp. was found to be maximum when 2% (v/v) inoculum was used and further increment in

inoculum volume did not show any stimulatory effect on lipase production in study carried out⁴². Whereas, maximum lipase production as achieved when 6% inoculum was inoculated in medium⁴³.

Likewise, 24 to 144 hr old bacterial inoculum were also inoculated to MSM medium to determine the effect of inoculum age on enzyme production and it was found that, 48 hr old bacterial culture was optimum to achieve maximum esterase production i.e. 0.55 U/mL (Fig 5). Bacterial inoculum older than 48 hr caused reduction in enzyme production may be because the bacterial culture reaches to its log phase after 48 hr which was followed by stationary and decline phase and thus the enzyme production by bacteria found to be higher in its log phase. Whereas, feruloyl esterase production from *Bacillus pumillus* was checked in the range of 3 to 46 hours and found that, maximum enzyme production was achieved at 226 hours incubation and reduced with further incubation⁴⁴.

Effect of carbon source

Additional carbon sources were added at 1% concentration to the medium to enhance the esterase production. From various carbon sources like, glucose, sucrose, fructose, cellobiose, cellulose and mannose, glucose enhanced the esterase production by bacteria upto 1.05 U/mL followed by fructose, sucrose, mannose, cellobiose and cellulose (Fig 6). In present study, bacterial culture able to degrade the pesticide has been isolated which is able to use pesticide as carbon source. May be bacteria utilize the pesticide as carbon and energy source in absence of any additional carbon source but in presence of glucose, the esterase production increased by utilizing it as carbon source that could affect the pesticide degradation ability of bacteria. Increment in lipase production by *Achromobacter xylosoxidans* TS2MCN and *B. thuringensis* TS11BP in presence of dextrose has been also reported³⁷.

Effect of nitrogen source

Various nitrogen sources like, ammonium sulfate, meat extract, urea, peptone, yeast extract were added at 1% concentration to the medium to check their effect on esterase production as nitrogen source is required by bacterium for enzyme production. Among all nitrogen sources, ammonium sulfate has been proved to be an optimum nitrogen source and gave maximum

activity i.e. 0.66 U/mL (Fig 7). Production of esterase enzyme by *B. coagulans* was enhanced in presence of ammonium sulfate³⁴. Presence of ammonium sulfate in medium triggers the production of esterase by *B. coagulans*. In many cases, inorganic nitrogen sources found to stimulate the esterase production over than organic nitrogen sources. Esterase production by *Bacillus circulans* was observed to be maximum in ammonium hydrogen carbonate supplanted medium compared to that in organic nitrogen sources⁴⁰. Stimulation in lipase production in presence of ammonium sulfate was also recorded⁴³.

Effect of MCP concentration

In present study, bacterial culture able to degrade the monocrotophos at 1000 ppm concentration has been isolated which is able to use pesticide as carbon source⁴⁵. When different concentration of monocrotophos was tested for maximum esterase production, *B. subtilis* KPA-1 was able to produce maximum esterase at 800 ppm concentration i.e. 1.50 U/mL/min. The isolated potent culture of *B. subtilis* KPA-1 was able to utilize the monocrotophos as carbon and energy source. Bacterium was able to utilize the MCP as carbon and energy source. So, MCP was only provided in nutrient medium. Initially, esterase production was very negligible by the bacterium when 100 ppm MCP was added. Esterase production was then increased gradually with increasing concentration of MCP upto 800 ppm followed by reduction in esterase production with further increment in MCP concentration as shown in figure 8 which may be due to substrate inhibition. Esterase production is depending on bacterial growth in presence of pesticide and the capacity of the bacterium to utilize the pesticide. There are some reports that suggest the influence of pesticide concentration on bacterial growth. The growth of cyhalothrin degradation by *Bacillus thuringiensis* strain ZS-19 in medium having different concentrations of cyhalothrin and observed that, the strain utilized the pesticide and grow maximally upto 800 µg/mL followed by reduction with increasing concentration⁴⁶.

CONCLUSION

In present study, ability of *B. subtilis* KPA-

1 to produce membrane bound esterase enzyme was determine which might be the reason behind the potential to degrade monocrotophos. The esterase enzyme produced by *B. subtilis* KPA-1 is a membrane bound enzyme and its production was found to be accelerated by optimization using OFAT approach.

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