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Statistical Optimization of Monocrotophos Biodegradation Using *Staphylococcus* Species

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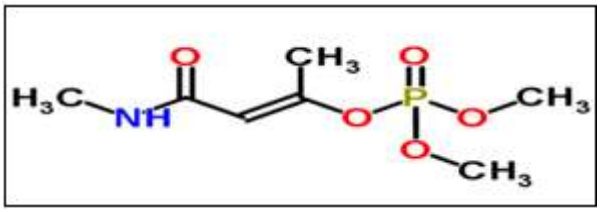
Abstract: ***Monocrotophos is both a systemic and control pesticide abundantly used for the protection of various cash crops. It is one of the highly hazardous organophosphate pesticides used and easily available in India even after being banned by WHO (World Health Organization). A potential monocrotophos degrading bacterial strain was isolated from soil by enrichment culture technique and identified as Staphylococcus spp. The bacterial growth and monocrotophos degradation were accelerated and increased by the isolate and it was capable of degrading about 54g. Culture conditions for degradation of the pesticide were statistically optimized by using eleven parameters and showed significant increase about 1.5 fold. The biodegraded sample showed greater reduction in toxicity as that of control on Allium cepathatproves bioremediation.***

Keywords: Biodegradation, Monocrotophos, *Staphylococcus spp*, Optimization

1. Introduction

Monocrotophos (MCP) is an organophosphorus insecticide and an acaricide, which is both a systemic and control pesticide useful for the protection of various cash crops such as cotton, sugarcane, groundnut, tobacco, maize, rice, soybeans, and vegetables by contact and stomach action from common mites, ticks, and spiders, pests. Proper understanding of the growth requirements of degrading organisms is necessary while using the pesticide degrading microbial system for the removal of pesticide residue and other products from contaminated systems. It is weakly sorbed by soil particles and easily penetrates into plant tissues due to its water-solubility.Generally organophosphorus (OP) compounds are ester or thiol derivatives of phosphoric, phosphonic or phosphor amidic acid. R1 and R2 are mainly the aryl or alkyl group, which can be directly attached to a phosphorus atom (phosphinates) or via oxygen (phosphates) or a sulphuratom ,Lee et al., 1990.

In some cases, R1 is directly bonded with phosphorus and R2 with an oxygen or sulfur atom (phosphonates or thionphosphonates, respectively). At least one of these two groups is attached with un-, mono- or di-substituted amino groups in phosphoramidates. The X group can be diverse and may belong to a wide range of aliphatic, aromatic or heterocyclic groups. The X group is also known as a leaving group because on hydrolysis of the ester bond it is released from phosphorus (Sogorb and Vilanova, 2002).



Structure of Monocrotophos

The mode of action of Monocrotophos includes inhibition of neurotransmitter acetylcholine breakdown. Acetylcholine is required for the transmission of nerve impulses in the brain, skeletal muscles and other areas. However, after the transmission of the impulse, the acetylcholine must be hydrolyzed to avoid over stimulating or overwhelming the nervous system. This breakdown of the acetylcholine is catalyzed by an enzyme called acetylcholine esterase. Acetylcholine esterase converts acetylcholine into choline and acetyl CoA by binding the substrate at its active site at serine 203 to form an enzyme substrate complex, thereby changing its structure and function. They bind to the serine 203 amino acid active site of acetylcholine esterase (LeJeune and Russell. 1999). The leaving group binds to the positive hydrogen of Histidine 447 and breaks off the phosphate, leaving the enzyme phosphorylated. Further reactions involve release of choline from the complex and then rapid reaction of acylated enzymes with water to produce acetic acid and the regenerated acetylcholine esterase(Lenz et al., 2007).

The regeneration of phosphorylated acetylcholine esterase is very slow and may take hours or days, resulting in accumulation of acetylcholine at the synapses. This inhibition causes convulsion, paralysis and finally death for insects and mammals (Ragnarsdottir, 2000).

The long persistence of the monocrotophos in the environment resulted in the bioaccumulation and potential toxicity towards non-target organisms due to which its use has been drastically reduced in the developed countries. This group of compounds has been replaced by the less persistent and more effective organophosphorus compounds. However, most of the organophosphorus compounds possess high mammalian toxicity. Among the organophosphorus compounds, glyphosate, chlorpyrifos, parathion, methyl parathion, diazinon, coumaphos, monocrotophos, fenamiphos and phorate have been used extensively and their efficacy and environmental fate have been studied in detail.

Pseudomonas aeruginosa, Clavibactermichiganense , Arthrobacteratrocyaneus, Agrobacterium radiobacter Bacillus megaterium and *Pseudomonas mendocina*are some of the most commonly used organisms in the degradation of Monocrotophos**.**(Jain Rachna et al., 2012).

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This study is mainly focused on Screening and isolation of the organism capable of degrading Monocrotophos, Identification and Characterization of the organism., Toxicity assessment, Optimization of the culture conditions for maximum degradation of monocrotophos.

2. Materials and Method

Isolation and Screening

The organism was isolated from the soil sample collected from agricultural field according to Jain Rachna et al., (2013) with minor modifications. For this, 1 gm of soil sample was serially diluted in 10 ml of 0.85% saline followed by dilution to appropriate concentration (10^{-5}). 100 µl of diluted sample was plated on nutrient agar and incubated at 37^{o}C for 24hrs.

The bacterial isolates were subjected to enrichment culture technique using Bushnell Haas media containing gradient of monocrotophos concentrations (0.5, 1, 1.5, 2, 2.5, 3%) as a sole carbon source and incubated at 37^{o}C (Stock monocrotophos 1% = 36 gms).

Identification and characterization

The selected efficient bacterial isolate was identified by morphological and biochemical tests. Colony characteristics of the isolate were tabulated after observing on nutrient agar which was incubated for 24 hrs at 37^{o}C. Bergey's Manual of Determinative Bacteriology was used as a reference to identify the bacterial isolate using different biochemical tests (Buchanan and Gibbons, 1984).

Estimation of Monocrotophos

An aliquot of test solution containing 0.2% to 2.0% of monocrotophos was taken in a 25 ml graduated tube and to it 1.0 ml of 1.0 molL^{-1} sodium hydroxide was added. The solution was kept for 30 mins at room temperature for complete hydrolysis. Then 1 ml of diazotized p-amino acetophenone was added and shaken thoroughly and kept at 0-5^{o}C for 15 mins for colour development. The initial yellow color turns to reddish-violet. The solution was then diluted to the mark with water and absorbance was measured at 560 nm against a reagent blank (Janghel et al., 2006).Same protocol was followed to estimate the amount of MCP degraded by the bacterial isolate in Bushnell Haas media containing gradient of monocrotophos concentrations (0.5, 1, 1.5, 2, 2.5, 3 %) on post incubation (96 hrs)

Intracellular esterase assay

The 72 hrs culture was subjected to centrifugation at 10,000 rpm for 15 mins at 4^{o}C. The supernatant was discarded and the pellet was treated with lysis buffer for one hour. The treated sample was centrifuged at 10,000 rpm for 15 mins. 1 ml of the supernatant was used as enzyme source. Esterase activity was monitored quantitatively according to the method of Gomori as modified by VanAsperen (1962). A typical assay mixture contained 5 ml of 0.5 mM substrate solution and 1 ml of enzyme extract. The reaction mixture was incubated for 15 mins at 27^{o}C and the reaction was arrested by the addition of 1 ml DBLS reagent. The reaction mixture was allowed to stand for 30 mins and the intensity of the color formed was measured at 600 nm. A calibration curve was prepared using 1-naphthol.

Optimization-Plackett-Burman design

For the present study the selected 11 factors were glucose, lactose, galactose, peptone, beef extract, ammonium nitrate, pH, inoculum size, manganese chloride, calcium chloride and monocrotophos. These variables were evaluated by 12 runs and the levels of each variable were determined.

3. Results and Discussion

Isolation and screening of microbes

Among the 3 soil samples collected, three isolates were selected as positive organisms which were capable of growing in the monocrotophos medium. Based on the growth, isolates were designated as isolate-1, isolate-2 and isolate-3. From the three isolated positive organisms, hyperactive strain was selected based on the complete degradation of monocrotophos in the BH broth. **Fig** 1 shows the growth of the hyperactive strain on nutrient agar. Isolate-1 was selected for further studies because of its superiority in degrading the monocrotophos when compared to other isolates.

Figure 1: Colonies of isolate -1 on nutrient agar

Similarly, in previous study, Priyanka Singh Baghel and Bhawana Pandey (2013)have studied the degradation of 1% MCP by inoculating the *Monococcus* and *Diplococcus* bacteria. The isolated bacteria can be used in bioremediation process to remediate the monocrotophos pesticide.

Rangaswamy and Venkateswaralu (1992) isolated 5 strains of *Bacillus spp*, of which MCP-3 strain was capable of completely degrading 0.5% monocrotophos. MCP was the most susceptible to bacterial degradation.

Identification and characterization

The selected hyperactive isolate-1 was subjected to identification and characterization using Gram's staining. The biochemical features of the isolate-1 is shown in figure 2 shows the results of biochemical tests which reveal the characteristics of *Staphylococcus spp.*, in comparison with Bergey's Manual of Determinative Bacteriology. Likewise, Jain Rachna et al., (2012) used Gram's staining and biochemical

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characteristics for the identification and characterization of organisms capable of degrading MCP.

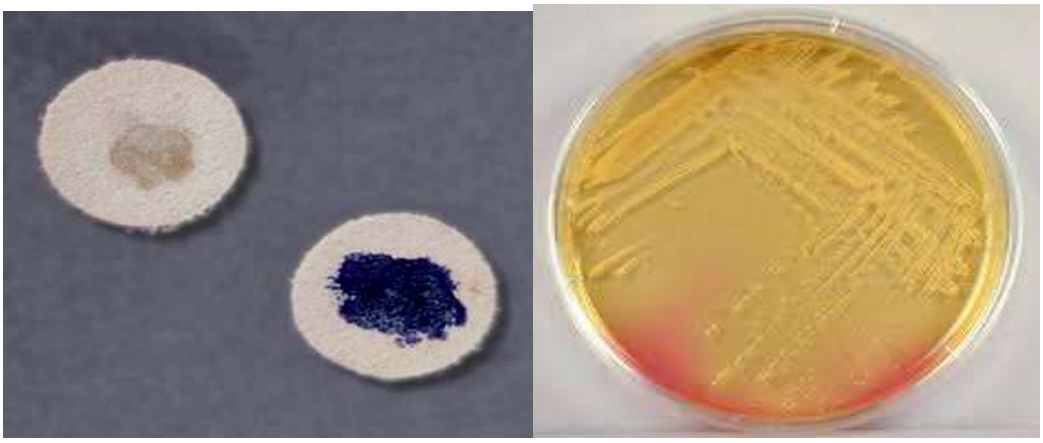

Figure 2: (a) Catalase positive **(b)** Mannitol Fermentation negative

Degradation of monocrotophos.

The amount of monocrotophos degraded by the isolated *Staphylococcus spp.*, were tabulated as in Table 1 and it shows the degradation pattern of monocrotophos in flask at various concentrations after 96hrs incubation.

Table 1: Degradation percentage of monocrotophos by *Staphylococcus spp.* after 96 hrs

Concentration of monocrotophos (%)	Amount of monocrotophos (g/100 ml) (a)	Recovered conc. of monocrotophos (b)	Recovery Percentage (c) (b/a x 100)	Percentage degraded (100-c)
Control	00	0	0.0	0.0
0.5	18	0	0	100
1.0	36	0	0	100
1.5	54	1.1	2	98
2	72	18	25	75
2.5	90	44	48	52
3	108	70	65	35

Figure 3 shows the degradation percentage of monocrotophos after 96 hrs incubation with the isolated *Staphylococcus spp.* It shows the complete degradation of about 50g and is capable of degrading up to 108g of monocrotophos, inferring that more the growth of the organism, higher the rate of degradation.

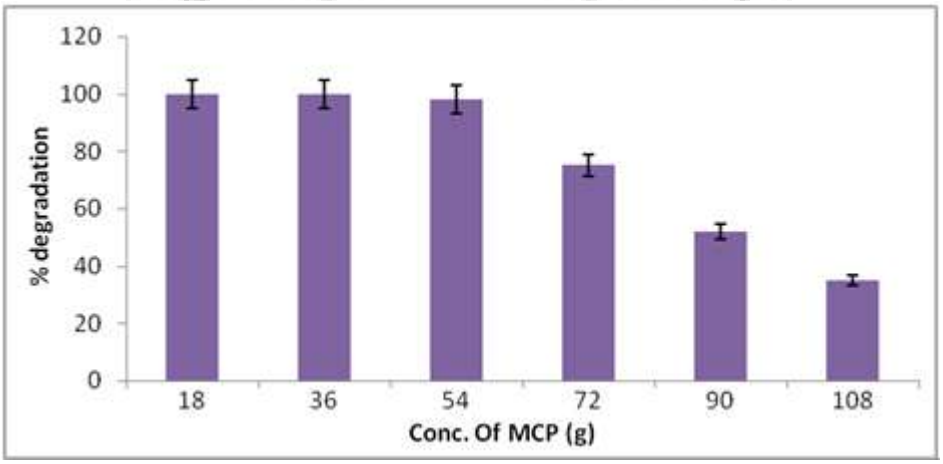

Figure 3: Degradation percentage of monocrotophos after 96 hrs incubation

According to Jain Rachna et al., (2012), the results of monocrotophos degradation kinetics are well correlated with its growth kinetics. After 10 days of incubation the percentage of degradation of monocrotophos was found to be approximately 90%, about 135 mg. Kai-zhiJia et al., (2006) reported increased levels of degradation of monocrotophos by *Paracoccus spp.*upto 16.78 mg/L. In comparison with the previous studies, our present investigation shows higher potential of the isolate in the degradation of monocrotophos.

Esterase assay

Table 2 shows the activity of intracellular esterase in the culture supernatants that were inoculated with the isolate

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Staphylococcus spp. up to seven days. The decreasing activity observed after 4 days may be due to the death/decline phase of the organism.

Table 2: Esterase activity

Days	1	2	3	4	5	6	7
Activity (U/ml)	8.0	9.4	10.2	11.4	9.6	6.0	5.0

Toxicity Assay

The application of *Allium* test enables us to give the general toxicity (indicated by root length of test plants). The mean root lengths of three replicates were estimated as general toxicity. The toxicity analysis results of water with monocrotophos and biodegraded sample after incubation for 96 hrs. The maximum root length measured was about 58 mm in the 4th day culture supernatant which was higher than 3rd and 5th day culture supernatant (26 and 52 mm respectively).

Figure 4 shows the *Allium* toxicity test with the positive and negative control along with biodegraded culture supernatant. Similarly, in previous study the comparative effect of pesticides was observed on *Allium* and mammalian cells indicating the test responds to the pesticides (Sarveshet al., 2009). In their study they have given consideration for 4th day measurement of root lengths. The 4th day concept may have arisen for the assessment of toxicity of pesticide sprays and formulations because the pesticides are applied at a particular time and there are intervals between the successive applications. So the impact of pesticide can be evaluated after 4th day from the time of exposure.

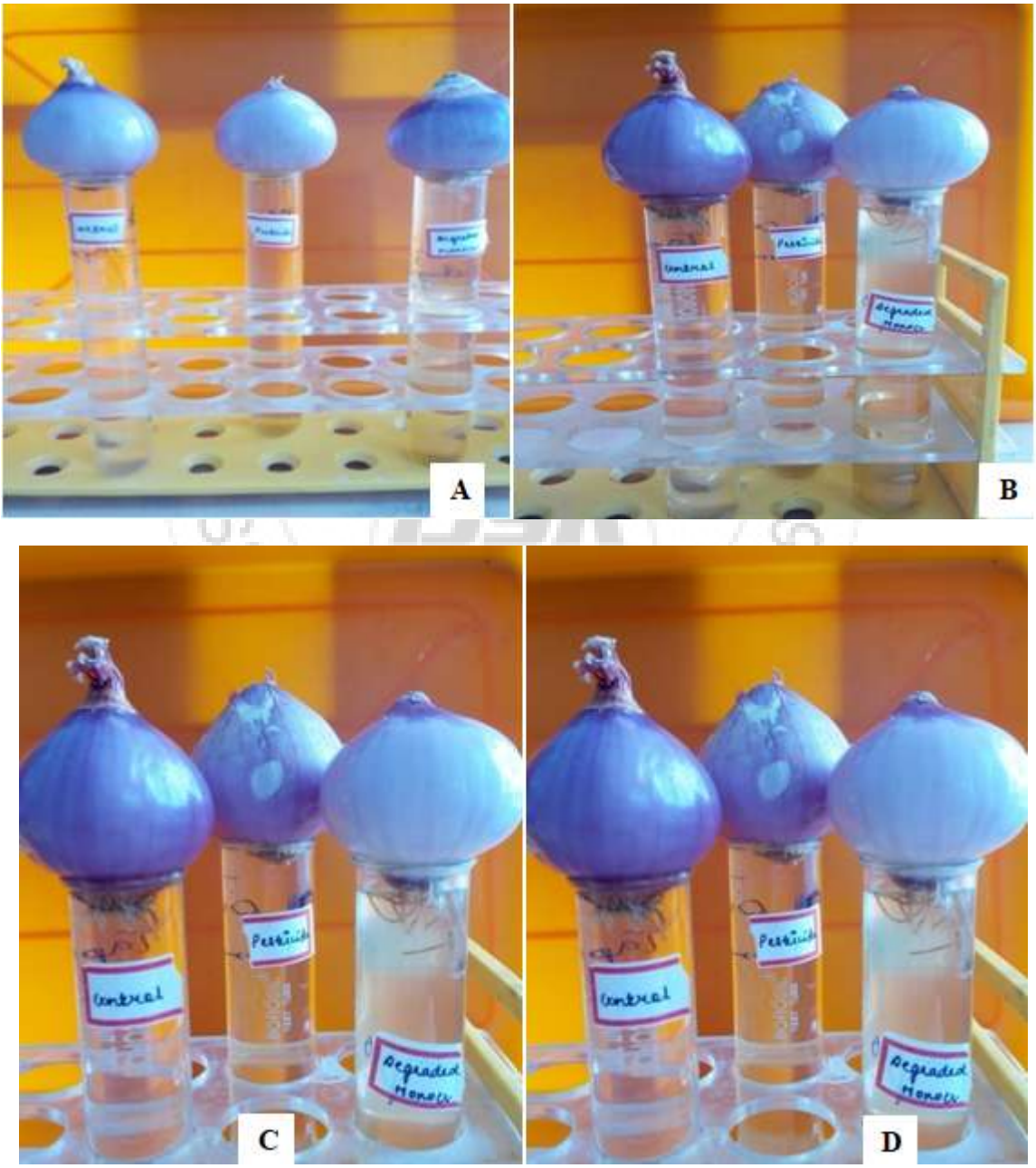

Figure 4: Developing root bulbs treated with degraded sample for 96 hrs.

Based on the present study the general toxicity between control and biodegraded sample, the biodegraded sample

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relatively showed lower toxicity. Hence it proves the usage of this isolated strain in the biodegradation of monocrotophos at higher concentration.

Optimization

It is a well known fact that the change in physical and chemical factors directly affects the growth of microorganisms. Hence, in this study the isolated strain was allowed to grow at different culture conditions and its growth was observed. Statistical methods have proved to be a powerful tool for the medium optimization. Table 3 shows the elucidation of medium components affecting esterase activity as examined by Placket-Burman design experiments. The data indicated that there was a wide variation of esterase activity in the 12 runs. This variation reflected the importance of medium optimization to attain higher activity.

Table3: Coded values of factors used in Plackett- Burman design

Expt	Factor levels (Coded)											Esterase Activity U/ml
	A	B	C	D	E	F	G	H	J	K	L	
1	-1	1	-1	-1	-1	1	1	1	-1	1	1	1.67
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.86
3	-1	1	1	1	-1	1	1	-1	1	-1	-1	1.12
4	-1	-1	1	1	1	-1	1	1	-1	1	-1	3.4
5	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.64
6	1	-1	1	-1	-1	-1	1	1	1	-1	1	2.56
7	1	-1	1	1	-1	1	-1	-1	-1	1	1	2.71
8	1	-1	-1	-1	1	1	1	-1	1	1	-1	1.70
9	1	1	-1	1	1	-1	1	-1	-1	-1	1	2.75
10	1	1	1	-1	1	1	-1	1	-1	-1	-1	1.15
11	-1	1	1	-1	1	-1	-1	-1	1	1	1	2.86
12	-1	-1	-1	-1	1	1	-1	1	1	-1	1	3.62

Table 4: Plackett-Burman design for screening variables for esterase activity

Factors	Unit	Code	Low level (-1)	High level (+1)	Effect
Glucose	g	A	0.5	1.5	Negative
Lactose	g	B	0.5	1.5	Negative
Galactose	g	C	0.5	1.5	Positive
Peptone	g	D	0.5	1.5	Positive
Beef extract	g	E	0.5	1.5	Positive
Ammonium nitrate	g	F	0.5	1.5	Negative
pH		G	6	8	Negative
Inoculum size	ml	H	1	3	Positive
Manganese chloride	mM	J	0.5	1.5	Positive
Calcium chloride	mM	K	0.5	1.5	Positive
Conc. of monocrotrophos	%	L	0.5	1.5	Positive

Figure 5 (Pareto chart) shows, the positive and negative influence of the screened variables on the esterase activity. The presence of high level of monocrotophos concentration, beef extract and magnesium chloride in the growth medium greately affects monocrotophos degradation positively. The factors which showed highest positive influence in the pareto chart analysis were consider for the future research.

Several studies have previously demonstrated the ability of bacterial biodegradation of pesticides (El-Sersy, 2001). Beef extracts has been identified as the best carbon source supporting maximum biodegradation of organophosphorous in certain earlier reports (Radha, 2005). Peptone and Beef extract has been the most commonly used nitrogen source for pesticide degradation processes with different organisms like *Pseudomonas luteola* (Hu, 1998), *Klebsiellapnuemoniae* (Wong and Yuen, 1996), *Bacillus* and *Clostridium sp*. (Knapp and Newby, 1995). Bacterial culture generally exhibit maximum degradation at a neutral pH value conditions (Pearce et al., 2003; Bhatt et al., 2005). Substantial differences in monocrotophos degradation appeared as a result of changing the variables concentration as done by Plackett-Burman experiment. This is one of the advantages of applying multifactorial experiments that consider the interaction of independent variables and provide a basis for model to search for the non linear nature of the response in short term experiment (Ravikumar et al., 2005).

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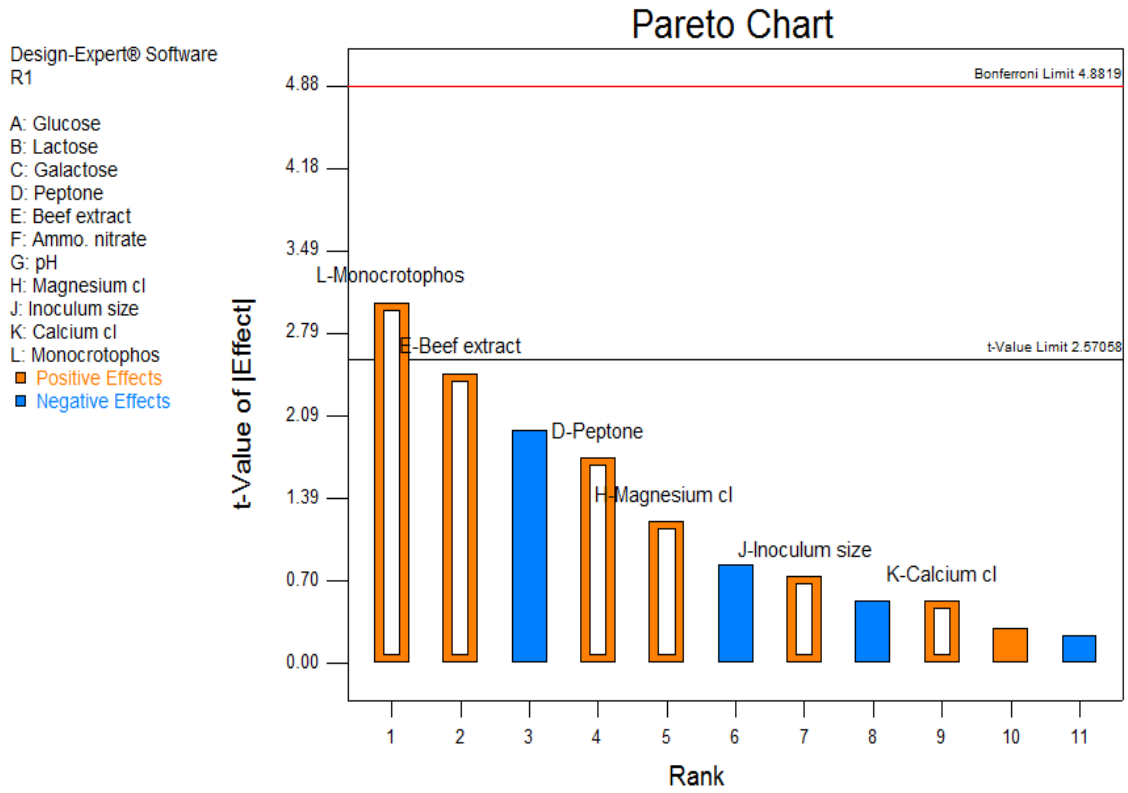

Figure 5: Pareto chart of eleven-factor effects on esterase activity

4. Summary

In the present investigation, a monocrotophos degrading bacterium was isolated from the soil samples of agricultural fields where the pesticide was used abundantly. Based on the morphological, physiological, biochemical tests and Bergey's manual of determinative bacteriology the isolate was identified as *Staphylococcus spp*. The potential of the bacterial isolate to degrade monocrotophos was found to be maximum of 54g (1.5%) after 4 days of incubation.

In order to determine the enzymatic pathway of degradation, the degraded sample was subjected to esterase assay and it was evident that the monocrotophos was degraded via intracellular esterase pathway. To increase the efficacy of degradation of monocrotophos by *Staphylococcus spp*., different culture conditions with 11 parameters were optimized using Placket-Burmann statistical method. Among the 11 parameters, monocrotophos, beef extract, peptone and magnesium chloride were found to have highest influence on the biodegradation of monocrotophos. Optimization results indicated that the organism was capable of utilizing monocrotophos more efficiently as the carbon source than any other sugars and acts as an inducer. The toxicity assessment of monocrotophos was studied using *Allium* test. Results were indicative of decreased toxicity after subjecting the pesticide to 96 hrs biodegradation by *Staphylococcus spp.*

5. Conclusion

It can be concluded that the bacterial isolate *Staphylococcus spp.* has the capacity to utilize and degrade monocrotophos at higher concentration and hence can be used for effective biological treatment of the hazardous pesticide. Thus, this investigation contributes by introducing a highly efficient bacterial strain for degradation of a hazardous pesticide like monocrotophos. Further investigations like strain improvement, culture conditions and molecular analysis may help the optimal usage of this isolate for the degradation of hazardous pesticides.

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