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Isolation of Metribuzin Degrading Soil Bacteria and Assessment of Their Growth in Response to Selected Physical Chemical Conditions

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Abstract.
Soil from designated farms with history of metribuzin use was screened for metribuzin degrading bacteria. Mineral salts media, incorporated with metribuzin as the sole carbon source was used to isolate the bacteria. Seven bacteria species were isolated and coded as follows; NZ453A, NZ454B, NZ453C, NZ543A, NZ543B, NZ8070 and NZ1110. Molecular characterization using 16sRNA was carried out to identify the isolated bacteria. They were identified to belong to groups of; *Pseudomonas sp., Burkholderia sp., Staphylococcus sp., Bacillus sp Arthrobacter sp., Bacillus sp.*,and *Planomicrobium sp*.,The effect of pH, temperature, nitrogen and phosphorous concentrations on growth of the isolated bacteria was assessed by optical density measurements in growth media. Generally, the bacteria recorded highest growth in alkali pH and temperature of 35^{o} C. Increased concentration of nitrogen and phosphorous showed a general increase in bacterial growth. Results showed a significant difference ($p < 0.5$) in growth among the isolated bacteria in the treatments used. The study revealed some of the physico-chemical treatments that can be used *invitro* for multiplication of metribuzin degrading bacteria for further development as bio degraders of metribuzin in contaminated sites. The information can also be used to create a favourable niche *insitu,* to enhance bacteria multiplication during bioremediation of metribuzin contaminated sites.
Keywords: Bacteria, Degrading, Metribuzin, Physico-chemical treatments

1.0 Introduction.

Nzoia River Drainage Basin is a major sugar production region in Kenya. Metribuzin herbicide is used in this region, for weed control. Residual metribuzin has been detected in soil and water in the Nzoia River Drainage Basin (Getenga *et al*. 2004, Ngigi *et al*. 2011). The excessive use of herbicides leads to accumulation of huge amounts of residues in the environment, thereby posing a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds in the food chain and drinking water (Smith and Walker 1989). Microbial metabolism is the major pathway for the removal and loss of metribuzin from soil (Pavel *et al*. 1999) and factors that influence microbial activity such as temperature, moisture and nutrient levels influence its degradation (Sharom and Stephenson 1976). The main physical factors which affect the activity of the soil biota are temperature, pH, soil moisture content, soil mineralogy and light (Killham1994) Temperature directly affects the activity of the soil biota by determining the rate of physiological activities such as enzyme activity, and indirectly by affecting physico-chemical properties such as diffusion and solubility of nutrients. The pH directly affects solubility of elements (Firestone *et al*. 1983; Flis *et al*., 1993). Essential minerals can become unavailable at extreme pH levels. Soil moisture affects the soil biota in two ways; biologically since water is essential for life and for enzyme activity and metabolism and, is a solvent for biological nutrients and other chemicals., Soil moisture affects soil temperature (water is good conductor of heat) and soil aeration (Killham1994).There is evidence that the soil environment can dramatically affect microbial degradation of pollutants. The soil pH, nutritional status, oxygen levels and temperatures vary in the environment and may not always be the optimal for growth, or enzyme production by bacteria (Singleton 2001).Phosphorus has a major ecological role in nature, because it is an essential element for microbes and because it is commonly the least abundant element compared to carbon (Wetzel 2003). Miettinen *et al.,* (1997) observed that addition of phosphorus (50 µg of PO^{4-} L^{-1}) increased microbial growth in fresh drinking water

produced from surface water or groundwater. In Kenya, the presence of metribuzin degrading bacteria in soil has not been demonstrated in agricultural systems, even though studies show presence of metribuzin in natural waters where the herbicide is used. The growth conditions and influence of physico-chemical parameters on bacteria populations are also not known, hence there is need to establish multiplication conditions of the bacteria for essential development for bioremediation.

2.0 Materials and methods

2.1 Study area

Nzoia Sugar Company is in Bungoma County of Kenya, which slopes from the foot of Mt. Elgon on the south eastern side. The county has a two-season rain regime, the long rains covering March to July while the short rains start in August to October. Temperature variations are very moderate ranging from 21 - 25°C during the year (Republic of Kenya 2014). Five major rivers drain the land (rivers Nzoia, Lwakhakha, Kuywa, Chwele and Khalaba), to Lake Victoria, East Africa's largest lake. The county is endowed with well-drained, rich and fertile arable soils. Five farms of Nzoia Sugar Company were identified for soil sampling to isolate metribuzin degrading bacteria. Details of these farms are given in Kariuki *et al*. (2015).

2.2 Isolation of potential metribuzin degrading bacteria

The soil for isolation of bacteria capable of degrading metribuzin was collected in five farms with a history of metribuzin application. The identification of isolates was based on colonial morphology, cultural and molecular characterization. The isolates were coded 'NZ' for Nzoia, followed by the farm number. The letter following farm number was the serial number of isolate. Response of seven isolates including NZ453A, NZ453B, NZ453C, NZ543A, NZ543B, NZ8070 and NZ1110 to selected physical chemical conditions such as pH, temperature, nutrients (nitrogen and phosphorous) concentration was investigated so as to act as guide to optimizing growth conditions in degradation experiments with these isolates**.**

2.3 Determination of growth Temperature.

The selected isolates were cultivated in growth media containing beef extract 5.0 g, peptone 10.0 g, sodium chloride 5.0 g, pH 7.0 and subjected to incubation temperatures of 20^{o}C, 27^{o}C and 35^{o}C to determine the effect of different temperatures on their growths. McFarland solution of optical density (OD_{600}) 0.2 was prepared (NCCLS 1990). The solution was used as a guide to prepare bacterial suspensions of 0.2 OD_{600} for each isolate as the initial concentration of bacteria cells, before incubation. Incubation was done for 48hrs and optical densities (OD_{600}) were measured using spectrophotometer (Thermofisher scientific, Genesys 10-S model, Germany), to establish best growth temperatures for the isolates and data was analysed by descriptive statistics.

2.4 Determination of optimum pH for growth of isolates.

The pH of the media was adjusted to 5.0, 7.0 and 9.0. Adjustments were done using dilute hydrochloric acid and dilute sodium hydroxide. Each isolate was cultivated into the media with adjusted pH and incubated at 35^{o}C ie optimum temperature from above experiment for 48 hrs. The optimal pH for the growth of the isolates was determined by measuring and recording of optical densities (OD_{600}) on spectrophotometer.

2.5 Determination of effect of Nitrogen concentration on growth of isolates.

Calcium ammonium nitrate (analytical grade) was used to adjust nitrogen concentration in the growth media. Concentrations of 5, 7.5 and 10 gL^{-1} were added to growth media.

Bacterial cells of OD_{600} 0.2 were prepared for each isolate as the start cells concentration before incubation. Each isolate was grown in each nitrogen concentration and optical densities (OD_{600}) of the cells were measured to determine growth performance of the isolates in the different nitrogen levels. The negative control consisted of bacterial cells of OD_{600} 0.2 in growth media, incubated for 48hrs at 35^{o}C as with the case with test samples. The difference in growth between the test and negative control was determined as the growth due to nitrogen effect as presented in the results.

2.6 Determination of effect of Phosphorous concentration on growth of isolates.

Di-ammoniun phosphate analytical grade was used to make phosphorous concentrations of 5, 7.5 and 10 g L^{-1}in the growth media. Bacterial cells of OD_{600} 0.2 were prepared for each isolate as the start cells concentration before incubation. Each isolate was grown in each phosphorous concentration and optical densities (OD_{600}) of the cells were measured to determine growth performance of the isolates in the different phosphorous levels. The negative control consisted of bacterial cells of OD_{600} 0.2 in growth media, incubated for 48hrs at 35^{o}C as the case with test samples. The difference in growth between the test and negative control was determined as the growth due to phosphorous effect as presented in the results.

2.7 Molecular characterization and identification

DNA was extracted using the phenol-chloroform method on seven isolates (NZ453A, NZ453B, NZ453C, NZ543A, NZ543B, NZ8070 and NZ1110) that showed ability to degrade metribuzin. The 16SrRNA gene was amplified by using polymerase chain reaction (PCR) with the universal primer pair of 16SF5'AGAGTTTGATCCTGGCTCAG 3' and 16SR5'GTACGCTACCTTGTTACGAC 3' The conditions for Polymerase chain reaction (PCR) were: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, at 48°C for 1 min, and 72°C for 3 min, and a final extension at 72°C for 10 min. PCR fragments were purified by the Gene Jet™ extraction Kit.

The 1445 base pairs of 16S rRNA gene sequence were determined at International Livestock Research Institute (ILRI), Kenya. Sequences were extracted from the database and aligned. Software DNAMAN 4.0 (LynnonBioSoft, Vaudreuil, Canada) and CLASTAL X 1.83 (Thompson *et al*., 1997) were used for sequence analysis and contrast. The Sequences were compared against available DNA sequences in GenBank.

3.0 Results and discussion.

3.1 Metribuzin degrading bacteria isolated from the soil.

Seven metribuzin degrading bacteria were isolated from the study farms soil. Their characteristics were determined by cultural, cell morphology and molecular characterization. Photomicrographs of the bacteria cultures on growth medium are shown on Figure I.

3.1.1 Cultural, cell morphology and Gram stain reactions of the isolates.

Bacterial cultural characteristics portrayed circular, rhizoid and root-like shapes as shown in table i. Five of the metribuzin degrading bacteria isolated, were gram positive while two were gram negative. Five were rod shaped while two were cocci.

3.2 Temperature treatment.

The growth responses of the isolates to different temperature treatments are represented in figure II. All isolates showed an increase in cell multiplication from initial optical density (OD_{600}) of 0.2 in all temperatures. Generally, NZ543A and NZ1110 showed highest growth in all temperatures of incubation compared to the other isolates. At 20^{o}C, NZ1110 showed highest growth recorded at OD_{600} value of 0.79 ± 0.02. Least performing isolate at 20^{o}C was NZ8070 with a mean OD_{600} of 0.54 ± 0.02. NZ543C had highest overall growth at 35 ^{o}C. Statistical difference in growth of individual isolates at different temperatures is shown in Table II. Generally, there was significant difference in growth of some of the isolates at different temperatures whereas others recorded no significant difference.

Metribuzin-degrading soil bacteria from Nzoia Sugarcane farms portrayed different growth temperatures. Six of metribuzin degrading bacteria from Nzoia Sugarcane farms had highest growth at 35 °C, while one had highest growth at 20 ^{o}C. Generally, there was significant difference in isolates' growth in 20 ^{o}C, 27 °C and 35 °C, temperatures. This indicates capability of the isolates to multiply and degrade metribuzin at different temperatures as the weather fluctuates in Nzoia cane farms. Petterson and Baath (2003) found out that soil bacteria community was better adapted to higher temperatures above 30 ^{o}C and it is confirmed in the study since more of the bacteria had highest growth in temperatures above 27 °C. Tamilselvan *et al.,* (2014) assessed growth of pesticide degrading bacteria in different temperatures and found the highest growth at 35 ^{o}C, followed by 25 ^{o}C and at 20 ^{o}C. These results were similar with the current study where the highest growth was at 35 ^{o}C, followed by 27 ^{o}C and at 20^{o}C.

3.3 Growth at different pH levels.

The effect of pH on growth of the isolates at different pH levels is shown in Figure III. As recorded pH 9 supported the highest growth in five of the seven isolates. NZ543A had highest growth of all at pH 7 with OD_{600} of 0.89 ± 0.21 while it had the lowest growth of all at pH 5 with OD_{600} of 0.38 ± 0.18. Generally, pH 9 reflected the highest pH at which highest growth took place in all isolates. At neutral pH of 7, NZ543A recorded relatively, the highest growth. Statistical difference in growth of individual isolates at different pH levels is shown in Table III. Generally, there was significant difference in growth of some of the isolates at different pH levels whereas others recorded no significant difference.

Five of the metribuzin degrading bacteria isolated from Nzoia sugarcane farms soil were recorded highest growth at pH 9 and two of them with highest growth at pH 7. The influence of pH on bacterial growth has been investigated previously. Baath and Arnebran (1995) reported that treatment of forest soils with lime and ash which resulted in pH changes from about pH 4 to 7 increased bacterial growths about five fold. Similarly, a study that included 19 different soils from areas with various land uses, spanning a pH range from 4 to 8, showed that there was an increase in bacterial growth with higher pH as measured by Leucine incorporation (Baath, 1998). Bacterial growth increased four-fold between pH 4 and 8 according to the study.

Todar (2008) observed that for most bacteria, there is an orderly increase in growth rate between the

minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing [H^+] on the rates of enzymatic reaction. Brock *et al.,* (2006) gave most bacteria growth range as neutral pH values of between 5 and 8, although some species can grow at more acidic or alkaline extremes as also observed in the study.

Tamilselvan *et al* (2014) assessed growth rate of some pesticide degrading bacteria in different pH media and found out that maximum growth recorded at pH 6, followed by pH 7, pH 8, and pH 5. The lowest growth rate was at pH 4. This differs from current study where the highest growth was at pH 9, followed by pH 7 and then 5. Both studies showed bacteria having lowest growth as pH became more acidic.

3.4 Growth of isolates in different nitrogen concentrations

The effect of different nitrogen concentrations on growth of the isolates is shown in figure IV. Generally, increased growth was observed in concentrations of 7.5 g L^{-1} and 10gL^{-1}in different isolates. Highest growth was observed in isolate NZ543A at concentrations of 7.5 g $L^{-1}$with mean OD_{600} of 0.85 ± 0.21. Isolate NZ543A had the lowest of all growths in nitrogen concentration of 10 g L^{-1} with OD_{600} of 0.26 ± 0.21. Statistical difference in growth of individual isolates at different nitrogen concentrations is shown in Table IV. Generally, there was significant difference in growth of some of the isolates at different nitrogen concentrations, whereas others recorded no significant difference.

Addition of nitrogen into growth media showed increased growth in all bacteria isolates though growth intensity varied among isolates. Cleveland *et al.,* (2002) observed that additions of nitrogen to nitrogen -poor systems did not result in consistent effects upon decomposer communities. In some cases, nitrogen did appear to stimulate microbial growth, but in many others its addition had no effect, or sometimes even a negative effect. The study was based on addition of nitrogen in soils and subsequent count of bacterial community biomass. Increase in growth in the current study could be due to adequate availability of other nutrients including carbon in the media with nitrogen further enriching the medium.

3.5 Growth in phosphorous concentrations

Fig. V shows effect of different concentrations of phosphorous on growth of isolates. The highest growth was in NZ1110 at concentrations of 10 g L^{-1} with OD_{600} of 0.83 ± 0.04 while the lowest was in NZ543A with OD_{600} values of 0.17 ± 0.04, at 10 gL^{-1}phosphorous concentration. Statistical difference in growth of individual isolates at different phosphorous concentrations is shown in Table V. Generally, there was significant difference in growth of some of the isolates at different phosphorous concentrations, whereas others recorded no significant difference.

The isolates under study showed higher growth in high concentrations of inorganic phosphorous. Out of the seven isolated soil bacteria, five had highest growth in phosphorous concentration of 10 g L^{-1} and two had highest growth at phosphorus concentration of 7.5 g L^{-1}. Phosphorus has a major ecological role in nature, because it is an essential element for microbes and because it is commonly the least abundant element compared to carbon (Wetzel 2003). Miettinen *et al.,* (1997) observed that addition of phosphorus (50 µg of PO^{4-} L^{-1}) increased microbial growth in fresh drinking water produced from surface water or groundwater. From that study it was evident that, addition of phosphorus to drinking water samples greatly increased the growth of heterotrophic bacteria. This was also observed in the current study, where addition of phosphorous in nutrient broth increased the isolates growth. In bio-stimulation studies nutrients such as phosphorus and nitrogen are required to speed up biodegradation of pollutants (Adams *et al.,* 2015). This means that when nitrogen and phosphorous based fertilizers are added in the farms, there is a possibility that bacteria would multiply in numbers and hence enhance degradation of residual pesticides that may exist in the soil, but generally higher growth was in higher concentrations of phosphorous than in nitrogen.

3.6 Molecular description of isolated bacteria.

The study revealed that the isolates were similar to previously identified xenobiotics degrading bacteria. Table VI shows a summary of the isolate code, name of the identified bacteria, their accession numbers assigned by genebank and other details. Isolate NZ453C showed 97 % similarity to *Pseudomonas putida*; NZ8070, had 97 %, similarity to *Planomicrobium flavidum*; NZ1110 was 98 % similar to *Bacillus amyloliquefaciens*; NZ453B, 98 % to *Burkholderia cepacia;* NZ543C, 98 % to *Arthrobacter globiformis*; NZ453A had 97 % similarity to *Staphylococcus sciuri* and NZ543A 99 % to *Bacillus pseudomycoides*. Gouma (2009) isolated nineteen *Bacillus sp* and four *Pseudomonas sp* which exhibited tolerance to metribuzin and a mixture of other pesticides. According to Abo-Amer (2012), *Pseudomonas sp*. is the most efficient bacteria genus for the degradation of toxic compounds. Zhang *et al* (2014) reported isolation of *Bacillus sp.* which degraded 20mg L^{-1} of metribuzin, at a rate of 73 % in 120hrs. In the current study similar genera of bacteria were isolated. *Arthrobacter globiformis, Pseudomonas sp.* and *Bacillus sp.* are some of the bacteria described by Krutz (2010) as having triazine degrading genes. Metribuzin is a triazine and the current study has isolated similar genera of bacteria as

described by Krutz (2010). *Planococcus sp*. and *Staphylococcus sciuri*are found to be degraders of other compounds as described by Vennila and Kannan (2010) and Mrozik and Labuzek (2002), respectively. Vennila and Kannan (2010) screened *Planococcus sp*. for hydrocarbon degradation and bioremediation and the bacteria showed capability to utilize kerosene as carbon source in minimal medium. Biological treatment of the refinery effluent with the *Planococcus sp.* reduced the oil and grease sulphide content to about 91.2% and 28% respectively on the 4th day of incubation. The identified bacteria species, and their accession numbers and established growth conditions are shown in table 4.

3.7 Summarized established growth conditions of the bacteria and their accession numbers.

The optimal growth conditions of isolates in different concentrations of phosphorous, nitrogen, at different pH and temperature as determined by highest optical densities (OD_{600}) are summarized in table VI. Five isolates showed optimal growth conditions in temperature of 35 º C; while one each had optimal growth at 20 and 27 º C. Five of the isolates had optimal growth at pH 9, two isolates at pH 7. In phosphorous, five isolates had optimal growth at a concentration of 10mg/L, while two at a concentration of 7.5mg/L. None had optimal growth at 5g L^{-1}. In nitrogen, three isolates had optimum growth in concentrations of 10 g $L^{-1}$and 7.5 g L^{-1}. Only one isolate showed optimal growth in 5 g L^{-1} of nitrogen.

4.0 Conclusion

This study has revealed the optimum growth conditions for bacterial isolates with potential to degrade metribuzin. There is a need to monitor metribuzin degradation with each of the isolates to find out the best degrader and also to establish metribuzin degradation pathway with the aim of coming up with cultures or culture conditions that can be utilized in bioremediation of soils in Nzoia sugar cane farms.

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Conflict of interest.

All authors declare that there is no conflict of interest either financial/commercial or any other form of interest. This information has not been published elsewhere and is devoid of plagiarism.

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Table I Summary of Cultural, cell morphology and Gram stain reactions of the isolates.

Isolate	Cultural characteristics	Gram reaction	Cell morphology
NZ453B	Circular-white fast growing	Negative	Rods
NZ453C	Circular white colonies	Negative	Rods
NZ453A	Circular-pinpoint-size yellow colonies	Positive	Cocci
NZ543A	Rhizoid white colonies	Positive	Rods
NZ543B	Circular - white - mucoid colonies	Positive	Rods
NZ1110	Root-like spreading white colonies	Positive	Rods
NZ8070	Circular white colonies	Positive	Cocci

Table II Statistical difference in growth (OD_{600}) between isolates at different temperatures using separation of means by LSD

	Isolates						
Temperature	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
20^0 C	0.647^a	0.665^a	0.593^a	0.700^a	0.659^a	0.791^a	0.549^a
27^0 C	0.671^a	0.676^a	0.535^a	0.826^b	0.678^a	0.812^a	0.553^b
35^0 C	0.717^b	0.739^b	0.514^a	0.875^c	0.736^b	0.801^a	0.793^c

Same letters denote no significant difference on bacteria growth at different temperatures $p \leq 0.05$

Table III Statistical difference in growth (OD_{600}) between isolates at different pH levels using separation of means by LSD.

	Isolates						
pH	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
pH 5	0.554^a	0.511^a	0.425^a	0.378^a	0.786^a	0.536^a	0.443^a
pH 7	0.564^a	0.661^b	0.534^b	0.890^b	0.633^b	0.728^b	0.563^b
pH 9	0.784^b	0.791^c	0.655^c	0.506^c	0.761^a	0.734^b	0.473^a

Same letters in superscripts denote no significant difference in bacteria growth at different pH, at $p \leq 0.05$

Table IV: Statistical difference in growth (OD_{600}) between isolates at different nitrogen concentrations using separation of means by LSD

	Isolates						
Nitrogen	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
$5gL^{-1}$	0.739^a	0.728^a	0.546^a	0.695^a	0.701^a	0.874^a	0.713^a
$7.5gL^{-1}$	0.753^a	0.860^b	0.82^b	0.876^b	0.830^b	0.883^a	0.586^b
$10gL^{-1}$	0.875^b	0.841^b	0.76^b	0.255^c	0.765^a	0.843^a	0.574^b

Same letters in superscripts denote no significant difference on growth of bacteria at different nitrogen concentrations at $p \leq 0.05$

Table V Statistical difference in growth (OD_{600}) between isolates at different phosphorous concentrations using separation of means by LSD.

	Isolates						
Phosphorous	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
5 gL^{-1}	0.791^a	0.837^a	0.614^a	0.821^a	0.860^a	0.884^a	0.624^a
7.5 gL^{-1}	0.778^a	0.793^a	0.637^a	0.921^b	0.791^b	0.873^a	0.778^b
10 gL^{-1}	0.812^b	0.841^a	0.661^a	0.17^c	0.889^a	0.936^b	0.630^a

Same letters in superscripts denote no significant difference in bacteria growth at different concentrations of phosphorous, at $p \leq 0.05$

Table VI Summary of identified metribuzin degrading soil bacteria, their accession numbers and physico-chemical growth conditions that recorded highest growth in each bacterium.

Bacteria code	**Bacteria species**	**Accession number**	**Physical – Chemical growth conditions that recorded highest OD_{600}**			
			Temperature	**pH**	**Phosphorous**	**Nitrogen**
NZ453C	*Pseudomonas sp*	KX214668	35^oC	9	$10g^{-1}$	$10g^{-1}$
NZ453B	*Burkholderia sp*	KU937113	35^oC	9	$10g^{-1}$	$7.5g^{-1}$
NZ453A	*Staphylococcus sp.*	KX394232	20^oC	9	$10g^{-1}$	$7.5g^{-1}$
NZ543A	*Bacillus sp*	KX214666	35^oC	7	$7.5g^{-1}$	$7.5g^{-1}$
NZ543B	*Arthrobacter sp*	KX394231	35^oC	9	$10g^{-1}$	$10g^{-1}$
NZ1110	*Bacillus sp*	KX394233	27^oC	9	$10g^{-1}$	$10g^{-1}$
NZ8070	*Planomicrobium sp*	KX214667	35^oC	7	$7.5g^{-1}$	$5g^{-1}$

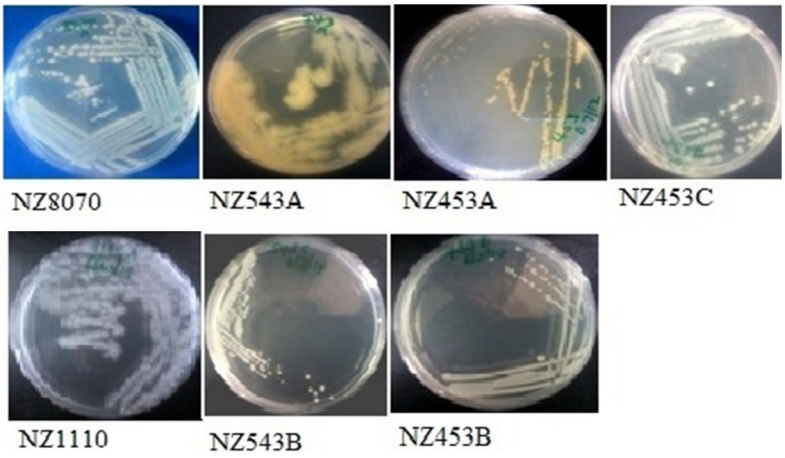

Figure I Photomicrographs of metribuzin degrading soil bacteria isolates.

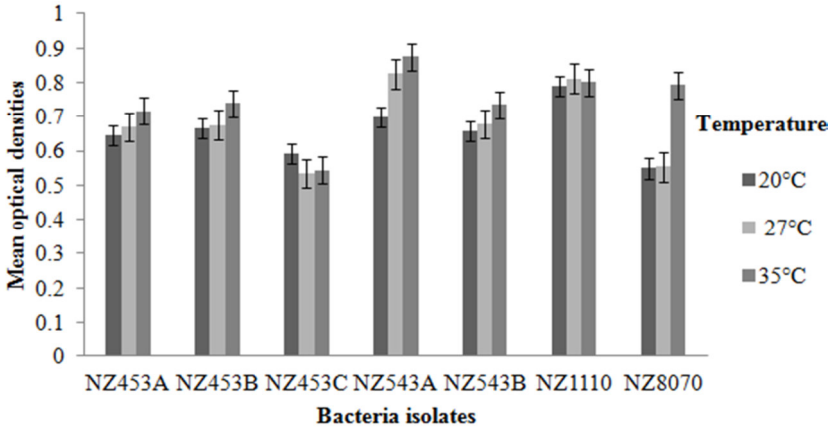

Figure II Column Charts showing effect of different temperatures on growth of isolates.

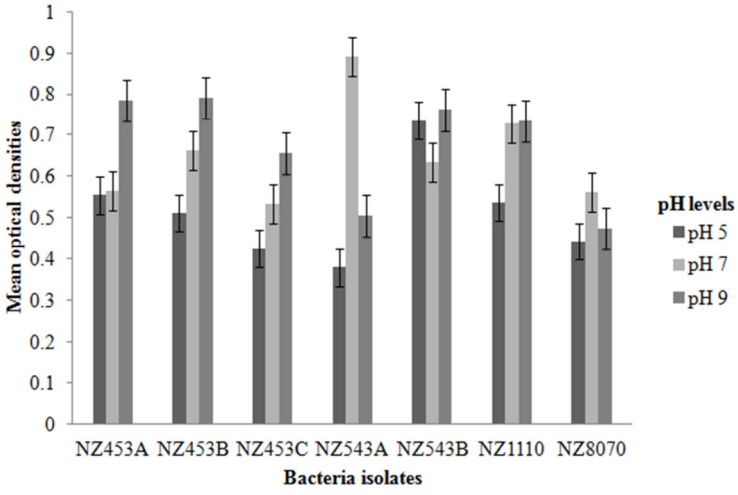

Figure III Column Charts showing growth of isolates at different pH treatments in growth media

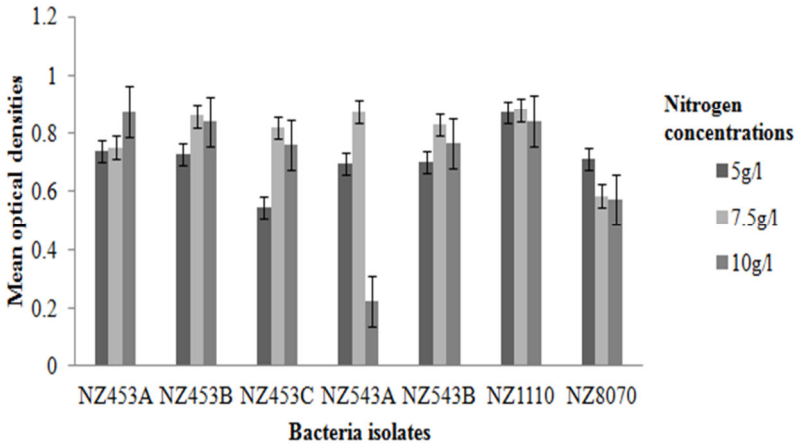

Figure IV Column Charts showing growth of isolates at different nitrogen concentrations on growth medium.

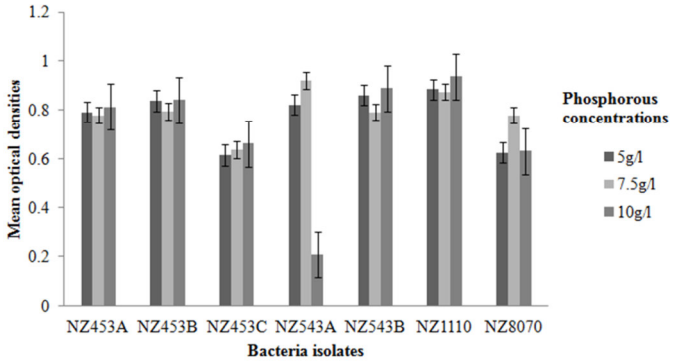

Figure V Column Charts showing growth of isolates at different phosphorous concentrations in growth medium