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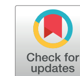
Research article

Immobilized cells of a novel bacterium increased the degradation of *N*-methylated carbamates under low temperature conditions



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ABSTRACT

Carbamates are synthetic pesticides, extensively used throughout the world due to their broad specificity against various insect pests. However, their enormous and inadequate use have made them a potential threat to the environment. At low temperature, degradation of carbamates becomes difficult mainly because of low biological activity. In the present study, we isolated a bacterial strain from a low temperature climate where the *N*-methylated carbamates are used for crop protection. The bacterium, was identified as *Pseudomonas plecoglossicida strain* (TA3) by 16S rRNA analysis. Degradation experiments with both free and immobilized cells in minimal salt medium indicated that the strain TA3 utilized carbaryl, carbofuran and aldicarb as both carbon and nitrogen source. TA3 can grow well at 4 °C and demonstrated the ability to degrade three carbamates (50 μgml^{-1}) at low temperature. The immobilized cells were found more efficient than their free cells counter parts. Immobilized cells has ability to degrade 100% of carbamates at 30 °C while 80% at 4 °C but incase of their free cells counter parts the efficiency to degrade carbamates was less which was 60% at 4 °C and 80% at 30 °C. TA3 free cellsextract also depicted high activity against all the three carbamates even at 4 °C indicating a possible enzymatic mechanism of degradation.

1. Introduction

N-methylated carbamates are the synthetic pesticides introduced into agricultural sector in 1950s and are still widely used for the management of a variety of pests os broad spectral nature (Tomlin, 2000). On the other hand, the carbamates are also an environmental concern due to their possible exposure to humans and other non-target organisms after their possible intrusion into food chain (EPA, 2006; Hughes and Wood, 2002). Therefore, their detoxification or removal is obligatory for protecting both the terrestrial and marine environments. A number of physical and chemical methods have been used for the elimination of these contaminants, but these conventional approaches have proved less effective, more troublesome and economically challenging as compared to the biological means of remediation (Theriot and Grunden, 2011; Tayabali et al., 2017; Camacho-Montealegre et al., 2019). Bioremediation is a promising technology for cleaning the sites polluted with pesticides and other lethal contaminants (Kumar, 2011; Zhang et al., 2017; Huang et al., 2018). This environmentally friendly and effective process takes benefit of the remarkable microbial capacity to either detoxify or alter the pollutants into harmless secondary compounds (Gratia et al., 2009; Holkar et al., 2016). Regardless of a well-demonstrated effectiveness of this process, climatic variations especially those of the temperature signify a major drawback in limiting the effectiveness of bioremediation process. At low temperature, the metabolic activities and growth of the indigenous microbes gets restricted in winter environment, resulting in poor biodegradation rates of pesticides and other pollutants (Aislabie et al., 2006). Low-temperature bioremediation and biodegradation of various organic pollutants in cold bionetworks and ecosystems is an outcome of the degradative capability of the native psychrophilic and psychrotropic microbial flora (Margesin and Feller, 2010). Microorganism have potential for use in bioremediation practices due to infrequent specificity and high catalytic proficiency of their enzymes. This includes the psychrotrophic bacteria, which can adapt themselves to a broad temperature range i.e. 0 °C to 30 °C. Many mesophilic bacterial strains having the

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ability to detoxify *N*-methylated carbamates have been isolated earlier (Naqvi et al., 2011, 2013; Fareed et al., 2017). However, carbamate degradation by the microbes adapted to the low temperature have not been described yet.

Over the recent past, immobilization techniques have received greater consideration. "Immobilization" is a broad term which describes a wide diversity of the entrapment of any cell (bacterial or fungal) as well as the biocatalyst linkage in any specific matrix (Zhang et al., 2004). The immobilized microbial cells offer easy handling of biocatalysts, which have been revealed to be of great benefit in a number of industrial practices for instance they are used in industries for citric acid and alkaloids production as well as for the degradation purposes and vice versa (Ha et al., 2009; Gotovtsev et al., 2015). It is very important to choose right entrapment matrix (Kocher and Mishra, 2009). Cell entrapment has been described to be the most efficient and cost effective method for the immobilization of cells. Various entrapment constituents have been in use which includes calcium alginate, agarose, polyacrylamide, agar-agar etc (Adinarayana et al., 2005; Rouf et al., 2017).

The immobolized microbial cells offer an auspicious potential strategy in almost all the bioremediation processes because of their better operational stabilities, higher biomass loading and enhanced biodegradation rates (Kulkarni and Chaudhari, 2007; Xue et al., 2017; Fu et al., 2018). They can be reused, thus reducing the expensive procedures of cell recycle and recovery. Furthermore, the immobilized cells can better tolerate toxic chemicals, solvents, heavy metals and high temperature and pH (Bayat et al., 2015). Immobilization methods have provided an alternative to overcome the problems faced by the use of free cells (Cassidy et al., 1996; Massalha et al., 2007; Farag et al., 2018). These problems include reduced mechanical strength, 5trouble in separation of bacterial biomass from effluents and low density of free cells (Massalha et al., 2007).

N-methylated carbamates are extensively use even on crops grow under low temperature and the residues accumulate for longer time as the environmental condiations does not prevail the growth of microorganisms. So, the present study was focused to isolate a psychrotrophic bacterial strain that can degrade *N*-methylated carbamates at low temperature in very efficient way. The bacterium was isolated and characterized and its cell free extract (CFE), immobilized cells as well its culture was use for the degradation of *N*-methylated carbamates at very low temperature. No such bacterium has been isolated till now that works at low temperature for the degradation of *N*-methylated carbamates. Moreover, Monod non inhibitory model was used to determine the growth kinetics of the free cells. These models in combination with the experimental work were used to enlighten the new characteristics of microbial structure and functions by offering a significant interpretation of the experimental results.

2. Materials and methods

2.1. Chemicals

Analytical grade *N*-methylated carbamates i. e aldicarb, carbofuran and carbaryl (99.9% pure) were purchased from Sigma Aldrich, USA. Synthetic oligonucleotides (primers) used in the present study were obtained from integrated DNA technologies, USA. All the samples were sequenced from Macrogen, Korea.

2.2. Sampling

Soil samples were collected for the isolation of psychrophilic bacteria, from the agricultural land with carbamate use history, from Gilgit, Baltistan, where, the temperature falls below freezing point during winter. Soil samples were collected in pre-sterilized bags and sealed immediately. The samples were freeze dried and stored at 4 °C to retain the biological activity of the soil microorganisms.

2.3. Culture media

Three different media were used in the present study. The MM (minimal medium with only 50 μgml^{-1} N-methylated carbamates were added as carbon and nitrogen source) was composed of NaCl (20 mg), K_2HPO_4 (48 mg), $Fe_2(SO_4)_3$ (0.1 mg), KH_2PO_4 (12 mg), $CaCl_2$ (40 mg), $MgSO_4.7H_2O$ (2 mg) per 100 ml sterilized distilled water. MMG (minimal medium containing glucose as carbon source while 50 μgml^{-1} N-methylated carbamates were added as nitrogen source) had similar composition as MM except with the addition of glucose (1%), and MMN (minimal medium containing nitrogen source while 50 μgml^{-1} N-methylated carbamates were added as only carbon source) where the media was amendded with NH_4NO_3 while all other compositions were same as in MM. The pH of each medium was adjusted to 7.0 either by usig the HCl or NaOH and then sterilized at 121 °C for 15 min.

2.4. Bacterial isolation and screening on different media

Carbamates degrading bacteria were isolated from soil by enrichment with carbaryl following the procedure as described earlier (Mohanta et al., 2012). Soil sample (50 g) was mixed with 100 ml sterilized distilled water in Erlenmeyer flasks (500 ml) and supplemented with carbaryl (10 μgml^{-1}) and incubated at 30 °C. After every two weeks, an equal amount of pesticide was added ill the final concentration reached 50 μgml^{-1}. Soil suspension (100 µl) from the serially diluted enriched culture was spread on MM, MMG and MMN agar plates containing carbaryl as their respective source and incubated at 30 °C for three days to get visible bacterial colonies. Afterwards, the bacterial colonies were streaked on above mentioned different media to check the efficiency of bacteria to utilize other *N*-methylated carbamates i.e. carbofuran and aldicarb. The bacterial strain showing the most prominent growth on all media was selected for further studies.

2.5. Strain identification and phylogenetic analysis

Taxonomic identification of the bacterial isolate was carried out by sequencing the 16S rRNA genes. EZ-10 spin column genomic DNA kit (Biobasic Inc, Canada) was used to extract the genomic DNA of the isolated strain. The amplified DNA was sequenced and compared with already existing sequences available in NCBI database. The phylogenetic tree was constructed on the basis of obtained blast results.

2.6. Enzymatic degradation

The specificity of enzyme was determined by using three different *N*-methylated carbamates as their substrates i.e. carbaryl, carbofuran and aldicarb. The cell free extract (CFE) obtained from the bacterium was used as an enzyme source. The hydrolysis of carbofuran and aldicarb was determined on the basis of rate of elimination of parent compound which was monitored spectrophotometerically at wavelength of 240 nm by using T80 UV/Visible spectrophotometer, Metller Toledo, USA. Carbaryl hydrolyzed in the presence of enzyme and converted into α-naphthol that reacts with fast blue BB salt forming brown adduct, was measured at λ_{max} 450 nm by spectrophotometer (Naqvi et al., 2009; Fareed et al., 2017).

Each enzyme assay was carried out at five different temperatures i.e. 4 °C, 10 °C, 15 °C, 20 °C and 30 °C. For the assay, reaction mixture (3 ml) was prepared, which contained 50 mM phosphate buffer, carbamate pesticide (50 μgml^{-1}) and 200 µl of cell free extract. All reactions were stopped by adding sodium dodecyl sulphate i. e SDS (10%). Before addition of SDS, 1% fast blue BB salt was added for carbaryl assay, that reacts with the hydrolysis product of carbaryl (α-naphthol) and forms brown adduct. Each reaction mixture was incubated for 60 min and the optical density was recorded after every 10 min. The decline in carbofuran and aldicarb concentration was observed at the wavelength of 240 nm which is proportionate to the elimination of parent compounds, whereas, the hydrolytic product of carbaryl (α-naphthol) was measured

at a wavelength of 450 nm (Fareed et al., 2017).

2.7. Immobilization of bacterial strain

Bacterial cells were entrapped in agar cubes following the previously described method (Woodward, 1988). 1 gram agar was dissolved in 100 ml of 0.9% NaCl solution and sterilized at 121 °C for about 15 min. The bacterial cells were obtained from overnight culture by centrifugation at 8,000 rpm for 15 min. The cell slurry was made by mixing cell pellets with 0.9% sterilized NaCl solution (1 ml). Finally, the bacterial cell slurry was added into 20 ml of molten agar solution, mixed well and then poured into sterilized petri plates. The petri plates were allowed to cool for few minutes. The solidified agar slab was cut into small cubes of equal size i.e. 10 mm^3 and placed in 100 mM sterilized phosphate buffer (pH 7.5) followed by incubation at 4 °C for 1 h to cure the cells. The phosphate buffer was removed and immobilized cells were preserved at 4 °C until further use. The viability of the immobilized cells was monitored by dissolving culture cubes in 100 mM sterilized phosphate buffer (pH 7.5) followed by spreading the aliquot (10 μl) on freshly prepared nutrient agar plates while incubating at 32 °C.

2.8. Biodegradation of carbamates by using free and immobilized cells

Batch experiments were conducted for biodegradation of carbaryl, carbofuran and aldicarb by using free and immobilized bacterial cells. Experiments were carried out at two different temperatures i.e. 4 °C and 30 °C for each *N*-methylated carbamate. In case of freely suspended bacterial cells, the cells were harvested at log phase and 240 mg of cells were inoculated in Erlenmeyer flask (500 ml) containig minimal salt medium along with particular carbamate (50 μgml^{-1}) except control i.e. without bacterial cells. Whereas, for immobilized cells, three agar cubes of approximately 1 g that contained 80 mg of bacterial cells, with dimensions of 10 mm^3 were added to each flask having minimal salt medium along with the carbamates, while, the agar cube without any bacterial cell served as control. Each experiment was carried out in replicates and incubated at 4 °C and 30 °C for 5 days on shaking incubators (250 rpm). Sampling was carried out aseptically after every 24 h for the analysis of residual carbamates.

2.9. Extraction of N-methylated carbamates from liquid media

The residual carbamates were extracted from the 3 ml supernatants by dissolving in equal volume of ethyl acetate with few modifications (Doddamani and Ninnekar, 2001). The samples were centrifuged for 15 min at 8000 rpm to remove the cells. The supernatants were extracted three times with 3 ml ethyl acetate by vigorous shaking for 5 min each. The top organic extract layer was pooled and passed through Na_2SO_4 (anhydrous) for the complete removal of water contents. The ethyl acetate was evaporated to near dryness under a gentle steam of nitrogen and the residual carbamates were reconstituted in 500 μl of acetonitrile. These samples were then stored at 4 °C until further analysis.

2.9.1. Quantification of residual carbamates

High performance liquid chromatography with UV-Vis detector (HPLC-UV-Vis) was used for the qualitative and quantitative determination of aldicarb, carbofuran and carbryl in reverse phase on a Perkin Elmer USA, 200 Series HPLC system consisting of a microprocessor controlled Perkin Elmer 200 Series eluent delivery quaternary pump and a fixed wavelength Perkin Elmer 200 Series UV–Vis detector. Samples were injected via a Rheodyne Model 7725i injector valve fitted with a 200 μl volume injector loop installed in a Perkin Elmer 200 Series Autosampler. The separation was achieved on a 150 × 4.6 mm reversed phase C18 column (Brownlee, Perkin Elmer) with a acetonitrile/water 35/65 (v/v) mobile phase at a flow rate of 1.0 $mlmin^{-1}$ under isocratic conditions. The column temperature was maintained at 25 °C in a Perkin Elmer Series 200 Column Oven. Data acquisition and processing were performed by Perkin Elmer software (TotalChrom v 6.3.2). Chromatographic determination was based on separation of pesticide analytes on C18 column and absorption at 200 nm.

2.10. Statistical analysis

SPSS 23 software was used for statistical analysis and one-way ANOVA and Duncan test was applied to compare the means of various treatments. The mean values were considered statistically significant with 95% confidence interval ($p < 0.05$).

2.11. Biodegradation kinetics of carbamates by free cells

The biodegradation rates of carbamates were determined by a number of mathematical models to define different parameters viz. substrate consumption by microbial cells, the bacterial cells growth and their decay during the biodegradation process. Monod in 1942 initially proposed the idea of microbial growth kinetics (Okpokwasili and Nweke, 2006) in the form of an empirical model (equation 1). In the present study, the Monod kinetics model (non- inhibitory) was applied to determine the bio-degradation kinetics of carbaryl, carbofuran and aldicarb.

$$\mu = \mu_{max}/(Ks + S) \tag{1}$$

where,

μ= specific growth rate,
μ_{max} = maximum specific growth rate,
S = growth limiting or residual substrate concentration,
K_s = half-saturation constant (mgL^{-1}), also called as affinity constant or substrate utilization constant. The kinetic constants i.e. maximum specific growth rate (μ_{max}) and half-saturation constant (K_s) of the bacterium was obtained by using hyper32 software.

2.12. Plasmid curing

Plasmids curing was achieved by following the method described by Brown (2000). Seed culture was prepared in LB broth supplemented with 1.5 g acridine orange as curing agent. After 16 h of incubation, 100 μl of culture was sub-cultured into fresh 15 ml LB broth containing acridine orange (1.5 g). The samples were then incubated for 3 days at 37 °C. After incubation, the culture (20 μl) was spread on LB agar plates to isolate single colony. The cured cells were then streaked on different minimal media containing carbamates to validate the plasmid curing with the control (the cells without plasmid curing).

2.13. Genetic diversity analysis by PCR amplification

Three major genes methyl carbamate degrading (*mcd*), carbryl hydrolase (*cehA*) and carbaryl hydrolase A (*cahA*), that are mostly reported for the degradation of *N*-methylated carbamates (Karns and Tomasek, 1991; Hashimoto et al., 2002, 2006) were amplified by using the standard protocol. The extracted DNA of the strain was amplified with specific primers designed by using NCBI tool "Primer design". Amplification was performed by using 2720 Thermal cycler of Applied Biosystems, USA. Each gene was amplified by itsspecific pair of primers i.e. for *mcd* (mcdF5′-ACGACCCATTTTCACGACGA-3′ and mcdR5′-CCGCTGCGA-TAGCAAAAGAC-3′), *cehA* (cehAF 5′-TGGACGGTCTTGAGAATGGC-3′ and cehAR 5′-ACGGATCGGTTTGTTTCCGA-3′), and for *cahA* (cahAF 5′-AGGCCTCTGGAGAGATCGTT-3′ and cahAR5′-TGTCCTCGGCGAAA-TATCCG-3′). After incubation for 4 min at 95 °C, 35 cycles of amplification (95 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min) were performed followed by 5 min extension step at 72 °C. The amplified product was run on 1% agarose gel along with DNA ladder to identify the product of required length.

3. Results and discussion

3.1. Bacterial isolation, screening and identification

Low temperature tolerant bacterium having ability to degrade *N*-methylated carbamates was isolated by enrichment technique. The bacterial strain TA3 was initially isolated by providing carbaryl as only source of nitrogen and carbon. Afterwards, the ability of TA3 to utilize aldicarb and carbofuran was also determined by providing these two pesticides as sole nitrogen and carbon source. TA3 showed an efficient growth on aldicarb and carbofuran.

The bacterial strain TA3 was identified by its 16S rRNA gene sequence analysis. The sequence of its amplified DNA had shown the highest degree of similarity i.e. 97% with *Pseudomonas* (accession number KX608546), therefor, it was identified as *Pseudomonas* strain TA3. The phylogenetic tree was constructed on the basis of blast results as shown in Fig. 1.

Earlier studies about carbamate degradation by different bacteria including *Pseudomonas* strains included mesophilic microorganisms, that do not show bioactivity at low temperature (Chaplamadugu and Chaudhry, 1993; Swetha et al., 2007; Naqvi et al., 2011). In the present study, a bacterium was isolated from low temperature environment that has the ability to degrade *N*-methylated carbamates. Although no carbamate degrading strain was isolated that works at low temperature but strains were isolated that degrade other organic compounds such as a psychrotroph was isolated that have the ability to degrade nitrobenzene at low temperatures was identified as *Pseudomonas putida* (Li et al., 2007). To the best of our knowledge, so far no bacterial strain for low temperature climate has been reported to degrade *N*-methylated carbamates.

Since, the low temperaure ecosystems are progressively exposing to hydrocarbons due to increased anthropogenic invasions. Therefore, many studies have focused on the bioremediation of hydrocarbons at low temperatures (Margesin and Feller, 2010). A number of the catabolic genes *"ndoB", "xylE", "todCI"* are accountable for the degradation of hydrocarbons like naphthalene and toluene. These genes capable of mineralizing dodecane ($C_{12}H_{26}$), hexadecane ($C_{16}H_{34}$), naphthalene $C_{10}H_8$, and Toluene (C_7H_8) have been identified in psychrotrophs and bacterial strains by using various molecular techniques in the cold ecosystems (Banerjee et al., 2016). Psychrotrophic hydrocarbon degrading bacterial strains like *Pseudomonas frederiksbergensis* and *Rhodococcus erythropolis* were able to utilize hydrocarbons at low temperatures (4 °C to 20 °C) (Megeed and Mueller, 2009). ***Arthrobacter psychrolactophilus,*** a low temperature adapted bacterial strain, was found to be proficient in

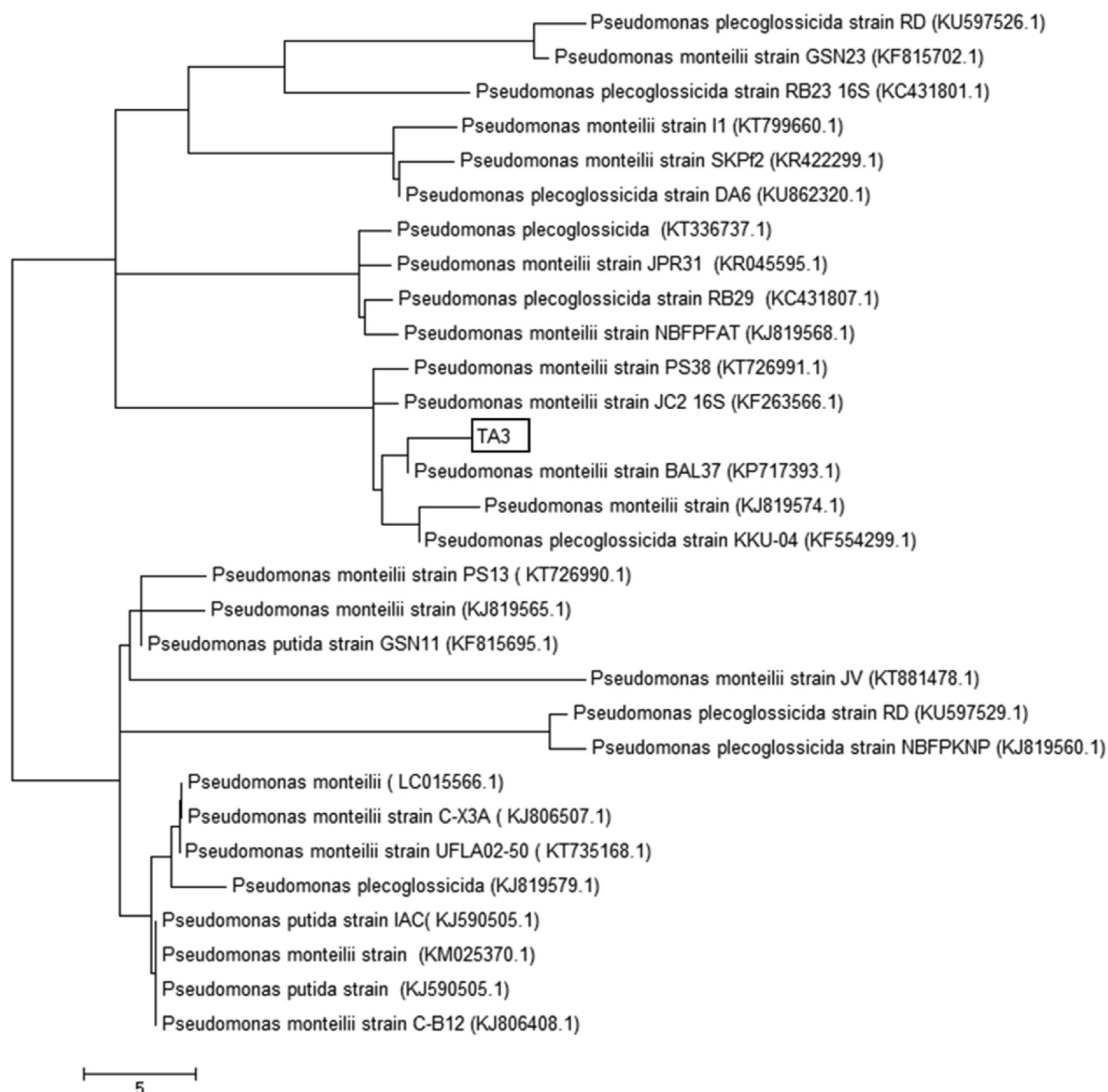

Fig. 1. Neighbor-joining phylogenetic tree of the representative bacterial strain TA3 and its related species based on the 16S rRNA gene sequences.

degrading organic contaminants in wastewater at 10 °C (Gratia et al., 2009). In arctic regions the role of bacteria as well as fungi has been recorded fro the degradation of plastics (Urbanek et al., 2017). Same type of findings were observed where indigenous microbial community play important role in the degradation of crude oil in arctic sea water (McFarlin et al., 2018).

3.2. Enzymatic degradation

The isolated bacterial strain TA3 was able to utilize *N*-methylated carbamates as evidenced by the growth of the bacterium on minimal media. Further confirmation was done by using its cell free extract (CFE) as source of enzyme against these carbamates. CFE obtained from *Pseudomonas* strain TA3, were able to catalyze aldicarb, carbofuran and carbaryl in given period of time at different incubation temperatures i.e. 4 °C, 10 °C, 15 °C, 20 °C and 30 °C, but the more pronounced enzymatic degradation was observed at 30 °C. The concentration of both carbofuran and aldicarb seemed to decrease with the passage of time as shown in Fig. 2a, Supplementary Fig. 1b–e, Fig. 3a and Supplementary Fig. 2b–e whereas, the concentration of α naphthol i.e. the hydrolytic product of carbaryl was increased with the passage of time at selected temperatures as shown in Fig. 4a and Supplementary Fig. 3b–e. The concentrations of these pesticides remained constant in untreated samples having no CFE. The results showed that the cell free extract can work at wide range of temperatures for potential carbamates degradation. Previously, many studies have also described the hydrolysis of the *N*-methylated carbamate pesticides by both CFE and purified enzyme (Chapalmadugu and Chaudhry, 1993; Doddamani and Ninnekar, 2001; Hayatsu et al., 2001; Hashimoto et al., 2002, 2006; Naqvi et al., 2009). An enzyme was isolated from the *Achromobacter* species having the ability to hydrolyse carbaryl, carbofuran and aldicarb efficiently (Derbyshire et al., 1987). Previously, Hayatsu et al. (2001) purified carbaryl hydrolase from *Arthrobacter* sp. RC 100 that can proficiently catalyzed XMC, metocarb, carbaryl and xylcarb. Carbaryl hydrolase purified from *Rhizobacterium* showed capability to hydrolyse carbamates including carbofuran and carbaryl (Hashimoto et al., 2002). According to our knowledge, it would be the first study where enzyme works at 4 °C and actively degraded all three *N*-methylated carbamates (Aldicarb, Carbofuran and Carbaryl).

3.3. Immobilization and biodegradation experiments

The degradation of each *N*-methylated carbamates (50 $\mu g ml^{-1}$) was carried out by using free as well as immobilized cells of TA3 in batch culture experiments at two temperatures i.e. 4 °C and 30 °C. The selection of these two temperatures is to evaluate the ability of TA3 strain at low as well as at ambient temperatures. As the strain was isolated from soil having low temperature, so it was expected that it will work at low temperatureas well as on ambient one. The viability and potential of agar-entrapped cells to grow on MSM containing carbamates was checked before performing the biodegradation experiments. The immobilized cells inside the agar cubes were not only viable but also grew proficiently on the agar plates containing minimal salt media.

The residual carbamates left in the liquid media after degradation were extracted after every 24 h and quantified by using HPLC. The results indicates a significant removal of these pesticides in all the treatments except control (untreated one). The immobilized cells were found more proficient in degrading carbamates in comparison with their free cells counterparts at both temperatures. Complete degradation (100%) was observed in samples inoculated with agar-immobilized cells at 30 °C, while at 4 °C, the degradation by the immobilized cells was 80–90%. On the other hand, the free cells degraded only 60% of the carbamates at 4 °C and upto 80% degradation was recordedby the free cells at 30 °C as shown in Fig. 5a–c.

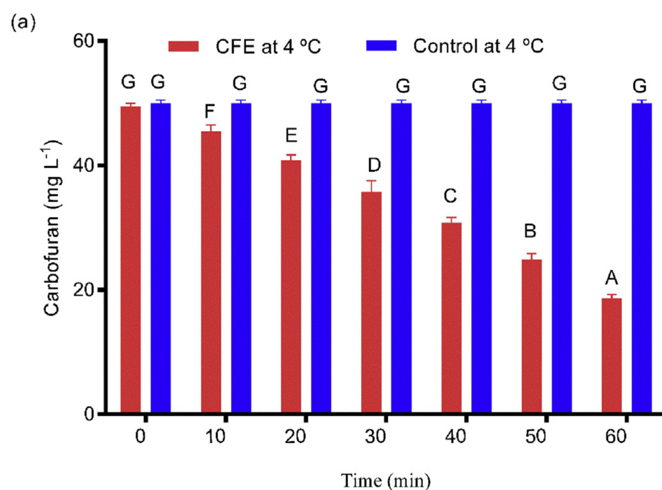

Fig. 2. Concentrations of carbofuran degraded after treatment with CFE obtained from TA3 at temperature (a) at 4 °C. Different alphabets show the significant differences ($p < 0.05$) between different treatments.

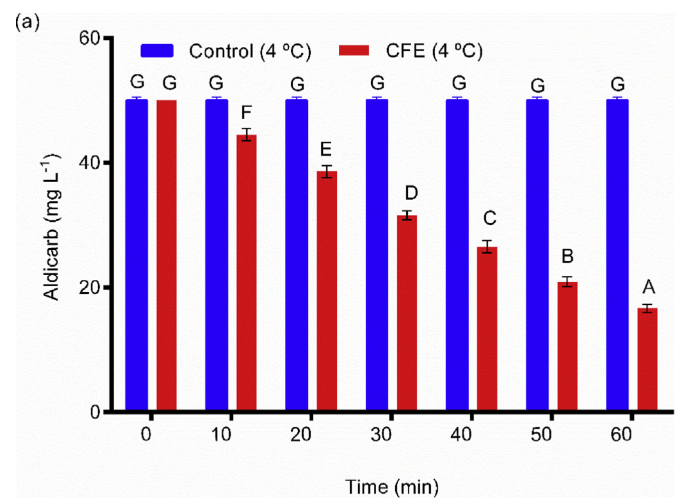

Fig. 3. Concentrations of aldicarb degraded after treatment with CFE obtained from TA3 at different temperature (a) at 4 °C. Different alphabets show the significant differences ($p < 0.05$) between different treatments.

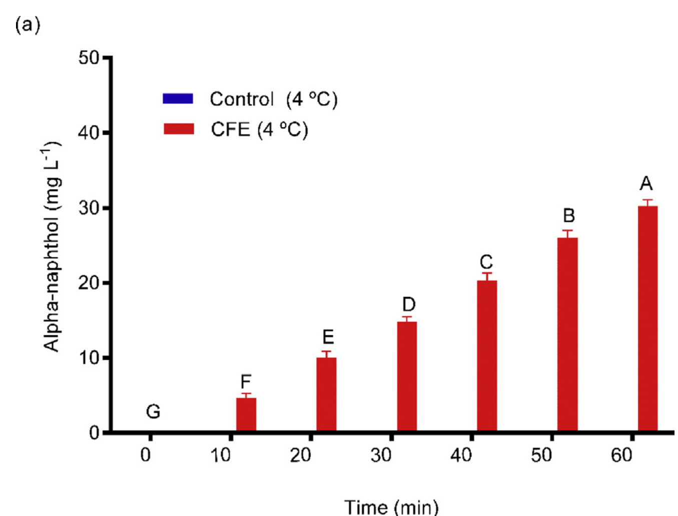

Fig. 4. Concentrations of alpha naphthol i.e. hydrolytic product of carbaryl degraded after treatment with CFE obtained from TA3 (a) at 4 °C. Different alphabets show the significant differences ($p < 0.05$) between different treatments.

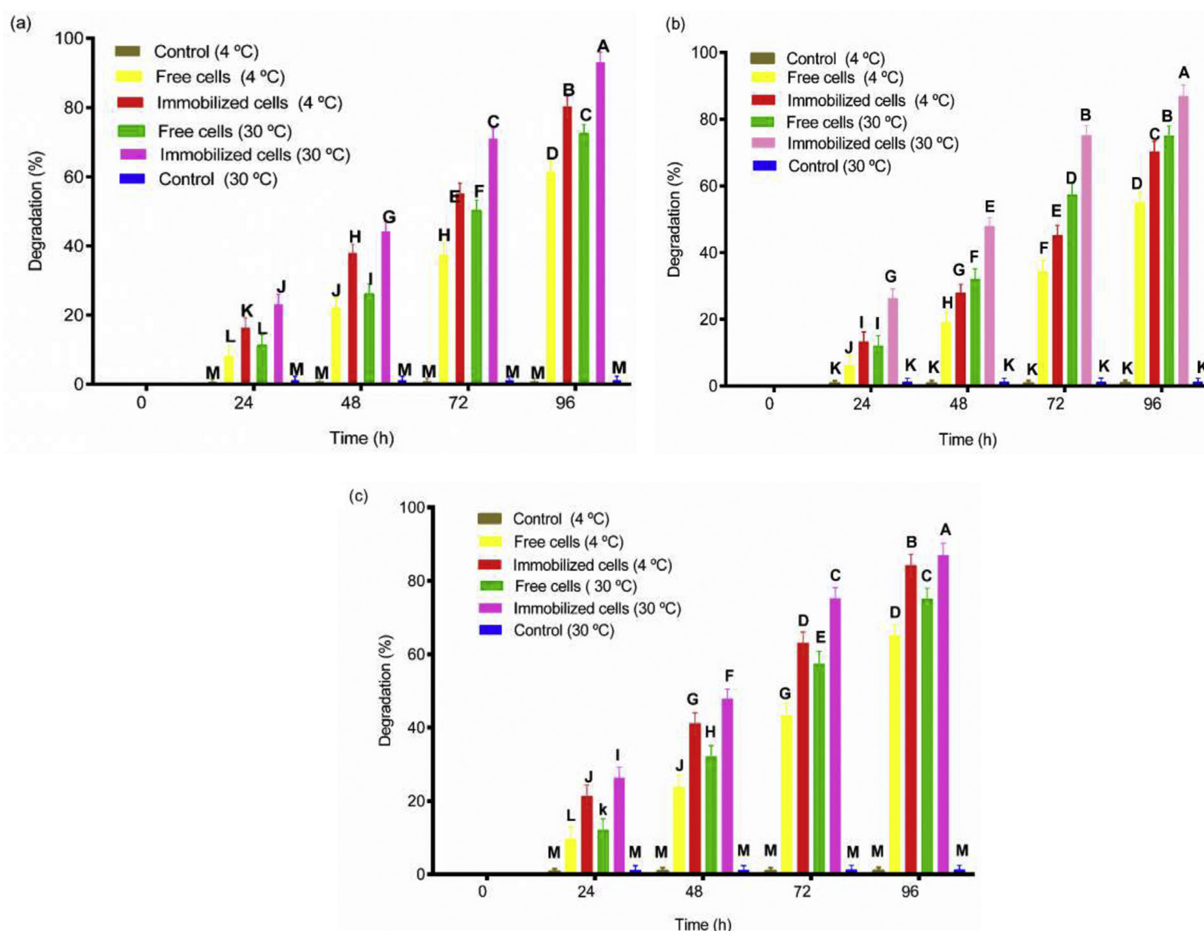

Fig. 5. Percent degradation at two different temperatures i.e. 4 °C and 30 °C (a) carbofuran, (b) aldicarb and (c) carbaryl. Different alphabets show the significant differences ($p < 0.05$) between different treatments.

The immobilized cells can be anticipated to have higher degradation efficiency due to their high mechanical strength and stability as compared to the free cells. Statistical analysis showed a significant difference between the degradation of both immobilized and free cells at both the temperatures as shown in Fig. 5a–c, respectively. The enhanced degradation of these pesticides by immobilized bacterial cells may also be due to their high density of cells inside the immobilized matric i.e. agar cubes. Furthemore immobilized cells needed less time to adjust themselves in new environment. The immobilized cells could better tolerated the concentration of all three pesticides which resulted in the higher degradation than their free cells. Similar findings were also been observed in our previous works where the immobilized cells of *Enterobacter cloacae* starin TA7 degrade carbamates faster as compare to their free cells counter parts (Fareed et al., 2017). Previously, Prabu and Thatheyus (2007) documented higher rates of acrylamide degradation by immobilized cells of *Pseudomonas aeruginosa,* where, acrylamide degradation started within 24 h of incubation, while, the free cells took 48 h to establish themselves. Similarly, higher rate of carbofuran phenol degradation was achieved by the immobilized cells of *Klebsiella pneumoniae* as compared with its freely suspended cells (Kadakol et al., 2011). In another study, the immobilized cells of *Pseudomonas putida* seemed to tolerate the higher concentrations of chlorpyrifos as compared to its free cells (Vijayalakshmi and Usha, 2012). Wang et al. (2003) reported that immobilized cells can degrade Di-n-methyl phthalate at a higher rate than the free cells. The enhanced degradation rate of crude oil was also observed in immobilized bacterial consortium (Xue et al., 2017; Farag et al., 2018).

3.4. Biodegradation kinetics

In the present study, the growth rate of *Pseudomonas* strain TA3 was determined by using different kinetic models for an initial concentration of 50 μgml^{-1} (50 ppm). Based on the experimental data, the Monod non-inhibitory growth kinetic model was found to have best fit. The kinetic parameters i.e. μ_{max}, $K_s$and μ_{max}/K_s for biodegradation of carbaryl, carbofuran and aldicarb at 4 °C and 30 °C were determined as shown in Table 1. The ratio of μ_{max}/K_s was considered to be most useful parameter, as it described the rate of substrate consumption. The higher values of μ_{max}/K_s indicated higher degradation or consumption rate of the substrate (Healey, 1980). In our study, TA3 showed higher value of μ_{max}/K_s (0.084 mgL^{-1}) at 30 °C than at 4 °C (0.04 mgL^{-1}) in case of carbaryl. This indicates that TA3 can degrade carbaryl at both the temperatures but the strain was more efficient at 30 °C. Similar pattern was observed for carbofuran and aldicarb, where, the value of μ_{max}/K_s were 0.049 mgL^{-1} and 0.039 mgL^{-1} at 30 °C and 0.03 mgL^{-1} and 0.029 mgL^{-1} at 4 °C, respectively. The kinetics reveals that the binding of strain towards all

Table 1
Kinetic parameters (μ_{max}, K_s and μ_{max}/K_s) of *Pseudomonas* sp. strain TA3 degrading *N*-methylated carbamates (50 μgml^{-1}) at 4 °C and 30 °C.

	Carbaryl		Carbofuran		Aldicarb	
	4 °C	30 °C	4 °C	30 °C	4 °C	30 °C
μ_{max} (h^{-1})	1.002	1.414	1.075	1.217	1.165	1.26
K_s (mg L^{-1})	24.53	16.72	25.05	24.81	29.17	32.01
μ_{max}/K_s (mg L^{-1})	0.042	0.084	0.030	0.049	0.0299	0.0393

three carbamates was strong when temperature was 30 °C, although TA3 has ability to degrade carbamates at low temperature i.e. at 4 °C but its binding towards all these carbamates is comparatively low. Previously, four models i.e. Monod, Blackman, Moser and Powell models were suggested for investigating bacterial growth rate while, Contois explained the accuracy of the Monod model for homogenous cultures and simple substrates rather than for heterogeneous cultures and complex substrates (Mahanta et al., 2014). Same model was also used for the detoxification of chlorpyrifos where he described efficacy of different bacterial strains against this pesticide by explaining μ_{max}/K_s values (Maya et al., 2011). Fareed et al. (2017) applied this Monod model in order to determine the growth kinetics of *Enterobacter cloacae* strain TA7. Recently, this kinetic model has also been used to describe the microbial growth as it defines the relationship between growth limiting substrate concentration and specific growth rate (Zaffar et al., 2018).

3.5. Plasmid curing

In order to elucidate the locatation of carbamate degrading gene i.e. their position either on plasmid or on the chromosomal DNA, TA3 cells were cured with acridine orange. After curing the bacterial cells grew efficiently on MSM using *N*-methylated carbamates as sole carbon 'C' and nitrogen 'N' source. These results proposed that the genes involved in the carbamate degradation were present on chromosomal DNA rather than on the plasmid. Hence, chromosomal DNA was isolated to amplify the carbamate degrading genes. Previously, Fareed et al. (2017) also reported that carbamates degrading genes located on chromosomal DNA rather than on the plasmid.

3.6. PCR amplification

Three genes (*mcd, cah A, ceh A*) involved in the degradation of *N*-methylated carbamates had already been reported (Karns and Tomasek, 1991; Hashimoto et al., 2002, 2006). In the present study, the DNA of the TA3 strain was amplified by using the specific primers of these genes. While performing the PCR for DNA ampilification with these pairs of primer, no product for *mcd*, *cah A* or *ceh A* was obtained, that clearly depicts that the strain TA3 has some other gene which is responsible for the degradation of these *N*-methylated carbamates.

4. Conclusion

A bacterial strain was isolated from soil samples collected from a low temperature climate and identified as *Pseudomonas plecoglossicida strain* TA3 by 16S rRNA. Both, free and immobilized cells of the bacterium were able to degrade *N*-methylated carbamates at 4 °C as well as at 30 °C. However, the immobilized cells performed more effectively as compare to their free cell counter parts. Furthermore, the crude extract obtained from TA3 can work in broad range of temperature from 4 °C to 30 °C which depicted the promising attitude of enzyme to degrade three different types of carbamates. Elucidation of such enzymes can be future prospect for the remediation of carbamates in low temperature environments as well as at higher temperature.

Declarations

Author contribution statement

Anum Fareed: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sania Riaz, Mazhar Iqbal, Raza Ahmed, Azhar Rashid: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ismat Nawaz, Azhar Hussain: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jamshaid Hussain: Analyzed and interpreted the data; Wrote the paper.

Tatheer Alam Naqvi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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