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Isolation and characterization of an acrylamide-degrading Antarctic bacterium

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Abstract: *The presence of acrylamide in the environment poses a threat due to its well known neurotoxic, carcinogenic and teratogenic properties. Human activities in various geographical areas are the main anthropogenic source of acrylamide pollution. In this work, an acrylamide-degrading bacterium was isolated from Antarctic soil. The physiological characteristics and optimum growth conditions of the acrylamide-degrading bacteria were investigated. The isolate was tentatively identified as Pseudomonas sp. strain DRYJ7 based on carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny. The results showed that the best carbon sources for growth was glucose and sucrose with no significant difference in terms of cellular growth between the two carbon sources ($p>0.05$). This was followed by fructose and maltose with fructose giving significantly higher cellular growth compared to maltose ($p<0.05$). Lactose and citric acid did not support growth. The optimum acrylamide concentration as a nitrogen source for cellular growth was at 500 mgl^{-1}. At this concentration, bacterial growth showed a 2-day lag phase before degradation took place concomitant with an increase in cellular growth. The isolate exhibited optimum growth in between pH 7.5 and 8.5. The effect of incubation temperature on the growth of this isolate showed an optimum growth at 15°C. The characteristics of this isolate suggest that it would be useful in the bioremediation of acrylamide.*

Key words: *Acrylamide, Biodegradation, Pseudomonas sp., Antarctica*
PDF of full length paper is available with author ***(*yunus@biotech.upm.edu.my)***

Introduction

Human activities are the main contributors towards pollution. Xenobiotics from industrial wastes and effluents have polluted almost all regions of the world. Even pristine areas especially in the polar regions have been affected (Ruberto *et al.*, 2005). The negative impacts of xenobiotics on various organisms have been documented (Ghosh *et al.*, 2006; Oyewo and Don-Pedro, 2006; Kumar *et al.*, 2007; Mondal *et al.*, 2007) and have prompted many researchers to search for solutions to remove these pollutants from the environment.

Acrylamide is the building block for the polymer, polyacrylamide. Uses of acrylamide include as sewage-flocculating agent (Myagchenkov and Proskurina, 2000), in the purification of drinking water, stabilising tunnels and dams, in agriculture in the form of hydrogel for hydroponic system and in the industry as adhesives (IPCS, 2003). However, commercial polyacrylamide preparation may be contaminated with its toxic monomer, acrylamide. Thus, regulations have been set on the amount of acrylamide that is present in polyacrylamide. For example, a limit of 500 ppm in polyacrylamide preparations is used in agriculture or water treatment. The widespread use of polyacrylamide has resulted in its ubiquitous presence in different geographical areas. It is known that all polyacrylamide from any sources will degrade to acrylamide under environmental conditions (Smith *et al.,* 1996) with the half life of acrylamide ranging from weeks to months in rivers (Brown *et al.,* 1980). Since acrylamide is a well known neurotoxicant, carcinogen and teratogen (Cherry *et al.*, 1956) its presence in the environment poses a hazard and its bioremediation must be sought. Many investigators have isolated bacteria from the environment capable of utilizing acrylamide/aliphatic-amides as the sole carbon and/or nitrogen source. However, acrylamide-degrading bacterium has never been isolated from polar region. Here we report on the isolation of the first acrylamide-degrading psychrotolerant bacterium from Antarctic.

Materials and Methods

Isolation of acrylamide-degrading bacterium and phenotype identification: Soil samples were a gift from the late Mr. Omar Pozan who collected the samples from Casey Station on the coast of Wilkes Land, in an area called the Windmill Islands (66.17°S 110.32°E) in Antarctica. Soils were collected randomly 15-20 centimetres (cm) beneath the surface using a sterile spatula and were placed in sterile screw-capped vials. The soil samples were placed in sterilized plastic bags and stored on ice during transfer from site to the laboratory. One acrylamide-degrading bacterium was isolated from the soil samples by enrichment culture using basal medium with 100 mgl^{-1} acrylamide as the sole nitrogen source. The growth and maintenance of the isolate was carried out according to the modified medium of Ciskanik *et al.* (1995). Briefly, the medium contained (per liter), 10 g of glucose, 0.5 g of $MgSO_4.7H_2O$, 0.05 g of $FeSO_4.2H_2O$, 100 mg l^{-1} acrylamide and 1 ml of the following trace elements; $ZnSO_4 \cdot 7H_2O$, 0.34 μM; $MnCl_2 \cdot 4H_2O$, 0.15 μM; H_3BO_3,

4.85 μM; $CoCl_2 \cdot 6H_2O$, 0.84 μM; $CuCl_2 \cdot 2H_2O$, 0.05 μM; $NiCl_2 \cdot 6H_2O$, 0.08 μM and $Na_2MoO_4 \cdot 2H_2O$, 0.123 μM. Subcultures were prepared by incubating the bacterium on a rotary shaker (200 rpm) for 48 hr at 25°C in 50-ml conical flasks containing the described medium. Isolates were maintained and grown in basal medium containing 100 mgl^{-1} acrylamide. Subcultures were inoculated into 150 ml volumes of the same medium and incubated with rotary shaking (200 rpm) for four days at 10°C. Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and molecular phylogenetics studies.

Monitoring of acrylamide degradation using HPLC method: Acrylamide degradation was monitored on an HPLC system (Agilent, 1100 series) consisted of a pump, a manual-injection system, an online degasser and a diode array detector. A pre-filtered [(0.45μm polytetrafluoroethylene (PTFE)] 20 μl sample was injected into Rheodyne™ sample injector and separation was performed on a column (Microsorb MV100-5 C_{18}, 4.6 x 250 mm, particle size 5 μm; Alltech Associates, Deerfield, Illinois, USA). Sample was eluted with filtered ultra pure water at a flow rate of 1 $mlmin^{-1}$. Detection was performed by monitoring the absorbance at λ = 196 nm using a reference wavelength of 360 nm (Caulfield *et al*., 2003). Acrylamide degradation was quantified by observing the decrease of the acrylamide amount during the experimental period. Degradation was determined by comparing the amount of acrylamide recovered from bacterial growth. Periodically, several ml aliquots from the acrylamide-degrading culture media was removed and divided into two portions. A portion is filtered using 0.45 μm polytetrafluoroethylene (PTFE) syringe filter to separate cells from the supernatant. The separated supernatant samples were analyzed to determine residual acrylamide level. At the same time, suitable serial dilutions of the other portion were carried out for bacterial plate count.

16S rDNA gene sequencing: Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using Biometra T Gradient PCR (Montreal Biotech Inc., Kirkland, QC). The PCR mixture contained 0.5 pM of each primer, 200 mM of each deoxynucleotide triphosphate, 1x reaction buffer, 2.5 U of *Taq* DNA polymerase (Promega) to achieve a final volume of 50 ml. The 16S rDNA gene from the genomic DNA was amplified by PCR using the following primers; 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' corresponding to the forward and reverse primers of 16S rDNA respectively (Devereux and Wilkinson, 2004). PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 25 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min; and a final extension at 72°C for 10 min. Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer. Sequence data were initially recorded and edited using CHROMAS Version 1.45. The resultant 824 bases were compared with the GenBank database using the Blast server at NCBI (Altschul *et al*., 1990). The analysis showed that this sequence to be closely related to *rrs* from Gammaproteobacteria. The 16S rRNA ribosomal gene sequence for this isolate have been deposited in GenBank under the accession number EF121821.

Phylogenetic analysis: A multiple alignment of 20 16S rDNA gene sequences closely matches strain DRYJ7 were retrieved from GenBank and were aligned using ClustalW (Thompson *et al.*, 1994) with the PHYLIP output option. The alignment was checked by eye for any obvious mis-alignments. Alignment positions with gaps were excluded from the calculations. A phylogenetic tree was constructed by using PHYLIP, version 3.573 (Retief, 2000), with *Bacillus subtilis* as the outgroup in the cladogram. Evolutionary distance matrices for the neighbour-joining/UPGMA method were computed using the DNADIST algorithm program. The program reads in nucleotide sequences and writes an output file containing the distance matrix. The model of nucleotide substitution is from Jukes and Cantor (1969). Phylogenetic tree was inferred by using the neighbour-joining method of Saitou and Nei (1987). With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps (Felsenstein, 1985) by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods called the MI methods (Margush and McMorris, 1981) using the CONSENSE program and the tree was viewed using TreeView (Page, 1996).

Statistical analysis: Values are means ± SE of at least three replicates. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance with post hoc analysis by Tukey's test (Miller and Miller, 2000). $p < 0.05$ was considered statistically significant.

Results and Discussion

Isolation of acrylamide-degrading bacteria: An acrylamide-degrading bacterium was isolated from Antarctic soil. Isolate J7 is a Gram-negative rod, is oxidase and catalase positive. The sequences obtained were from the direct PCR amplification of strain DRYJ7 gave a 16S rDNA gene sequence length of 824. The sequence was quite short but is enough to placed strain DRYJ7 within the Gammaproteobacteria. A low bootstrap value (< 50%) linked strain DRYJ7 to several *Pseudomonas* species indicating that the phylogenetic relationship of the strain to the species level of *Pseudomonas* could not be tied up (Fig. 1). Thus, the linkage of strain J7 is up to the *Pseudomonas genus* at this moment. Together with the Biolog identification system which gave the closest ID to *Pseudomonas stutzeri* with 95 percent probability, at least for now, strain DRYJ7 is assigned tentatively as *Pseudomonas* sp. strain DRYJ7. Many of the acrylamide-degrading bacteria reported in the literature is predominantly *Pseudomonas genus* with species reported to degrade acrylamide or aliphatic amide including *Pseudomonas aeruginosa* (Brown and Clarke, 1970; Prabu and Thatheyus, 2007), *Pseudomonas acidovorans* (Alt and Kirsch; 1975) *Pseudomonas putida* (Nawaz *et al*., 1998), *Pseudomonas chlororaphis* (Ciskanik *et al*., 1995), *Pseudomonas azotoformans*

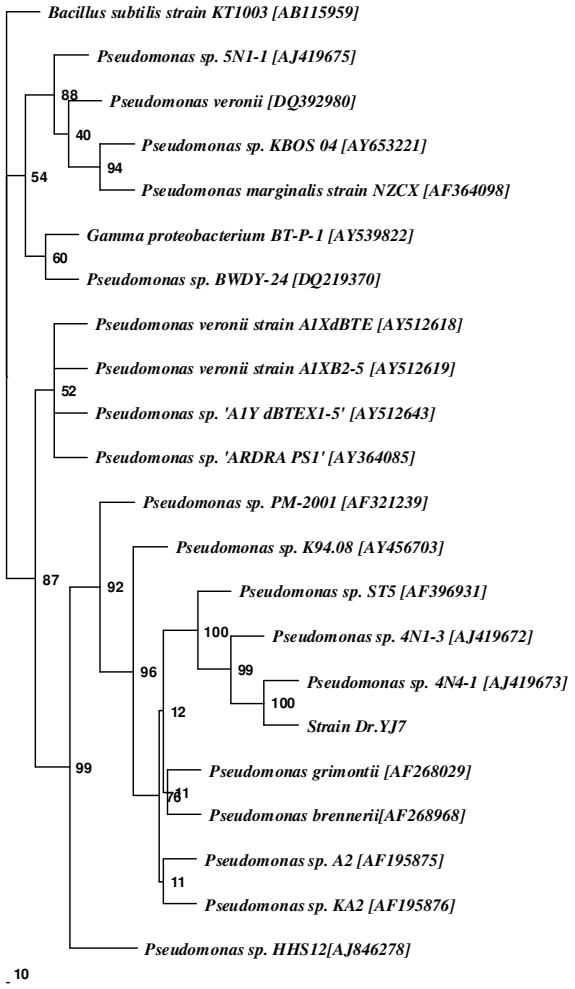

Fig. 1: A phylogram (neighbour-joining method) showing genetic relationship between strain DRYJ7 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. *Bacillus subtilis* strain KT1003 is the outgroup

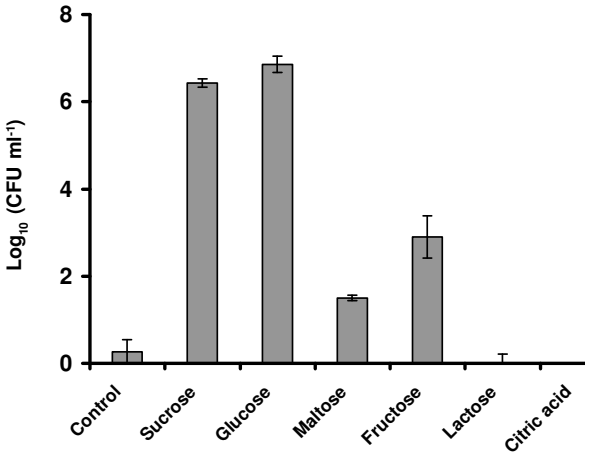

Fig. 2: Effect of different carbon sources (1% w/v) on the growth of Isolate DRYJ7. The error bars represent the mean ± standard deviation for three replicates

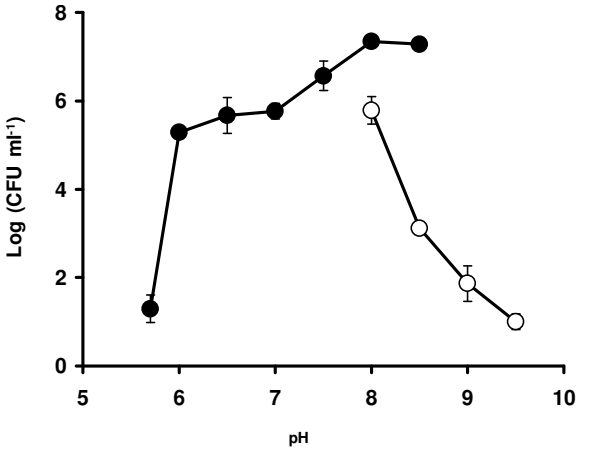

Fig. 3: Effects of pH on the growth of Isolate DRYJ7 using an overlapping buffer system consisting of phosphate (●) and carbonate (○) system. The error bars represent the mean ± standard deviation for three replicates

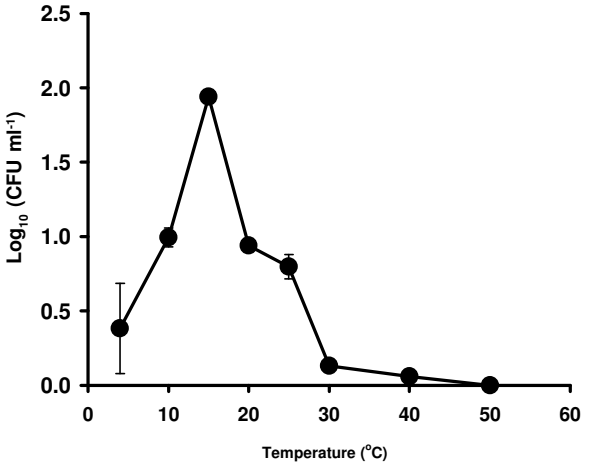

Fig. 4: Effect of temperature on the growth of Isolate DRYJ7 on acrylamide. The error bars represent the mean ± standard deviation for three replicates

(Komeda *et al.*, 2004a) and *Pseudomonas* sp. (Komeda *et al.*, 2004b).

Other acrylamide or aliphatic amide-degrading bacteria reported in the literature include Burkholderia cepacia *strain C (Novo* et al.*, 2003),* Brevibacterium *sp. R312 (Soubrier* et al.*, 1992),* Methylophilus methylotrophus *(Silman* et al.*, 1991), and* Helicobacter pylori *(Skouloubris* et al.*, 1997),* Pseudonocardia thermophilia *(Egorova* et al.*, 2004),* Rhodococcus rodochrous *(Kotlova* et al.*, 1999),* Rhodococcus *sp. (Nawaz* et al.*, 1998),* Thermococcus hydrothermalis *(Postec* et al.*, 2005),* Brevibacillus borstelensis *BCS-1 (Baek* et al.*, 2003) and* Klebsiella pneumoniae *NCTR1 (Nawaz* et al.*, 1996).*

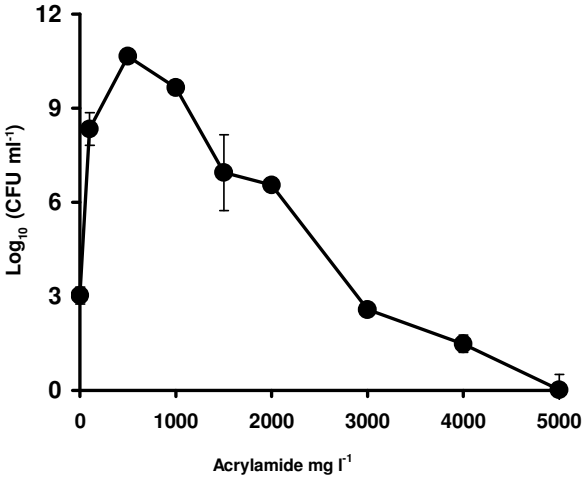

Fig. 5: Effect of different concentrations of acrylamide on the growth of Isolate DRYJ7. The error bars represent the mean ± standard deviation for three replicates

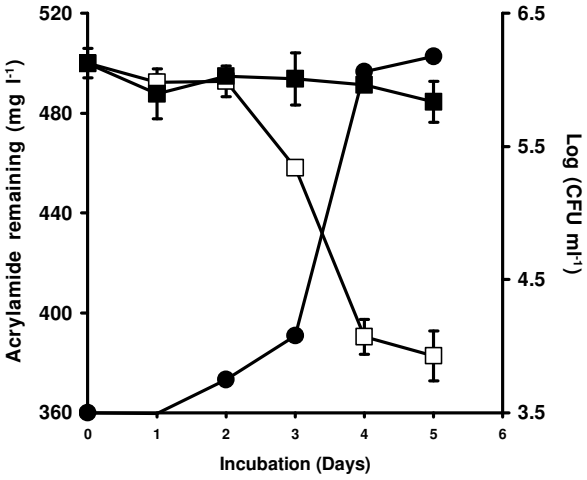

Fig. 6: Acrylamide degradation by isolate DRYJ7 in acrylamide enrichment culture (□), cellular growth (●) and abiotic control (■). The error bars represent the mean ± standard deviation for three replicates

The effects of carbon sources on growth of bacterium: The influence of difference carbon sources on the bacterial growth was investigated. The results (Fig. 2) showed that glucose and sucrose were the best carbon sources for growth with no significant difference in terms of cellular growth between the two carbon sources ($p>0.05$). This was followed by fructose and maltose with fructose giving significantly higher cellular growth compared to maltose ($p<0.05$). Lactose and citric acid did not support growth. The growth rate of bacteria usually increases when carbon sources and other nutrients are added to a minimal medium. Glucose is the universal carbon source that has been shown to enhance biodegradation of xenobiotics in general (King *et al.*, 1992). Previous studies in acrylamide-degradation have also identified glucose as the best carbon source when acrylamide is used as a nitrogen source. In several cases, 1 to 2% glucose appears to be the optimum (Nawaz *et al.*, 1996; Ciskanik *et al.*, 1995; Kotlova *et al.*, 1999). Aside from glucose, starch has also been used as a supplement for acrylamide degradation (Egorova *et al.*, 2004). In this study, the optimum concentration of glucose for maximum growth Isolate DRYJ7 was found to be between 1 and 2% (w/v) with lower growth at higher glucose concentrations.

Effect of initial pH: Identification of the pH optima for growth would help in designing effective bioremediation strategy (Davey, 1994). The effect of initial pH on the growth of Isolate DRYJ7 was studied at 10°C using an overlapping buffer system. This experiment was conducted in order to enhance the bacterial growth. The measurement of growth was carried out after 40 hr of incubation. Fig. 3 shows that isolate DRYJ7 grew at a relatively wide pH range from 6.0 to 8.5 with maximal growth attained from pH 7.5 to 8.5 with no significant difference in terms of cellular growth at these points ($p>0.05$). Growth dramatically decreased at lower and higher than the range. Phosphate was the optimum buffer for buffering medium during growth whilst carbonate gave significantly lower growth at pHs 8 and 8.5 ($p<0.05$). The requirement for neutral or near neutral pH for optimal growth on acrylamide is also shared by several acrylamide-degrading bacteria (Egorova *et al.*, 2004; Nawaz *et al.*, 1998 Komeda *et al.*, 2004b; Prabu and Thatheyus, 2007). The hyper thermophilic archaeon Thermococcus hydrothermalis cultivated in continuous culture yielded maximal growth rate at the optimal pH of 6.0 (Postec *et al.*, 2005). The bacterial growth is decreased at pH out of this range due to the lowering of the stability of the plasma membrane, inhibition of membrane enzyme and transport proteins (Booth, 1985).

Effect of temperature: The study of temperature optima for the growth of microbes would be very useful for bioremediation purposes (King *et al.*, 1992). Although generally it is not possible to change temperature when conducting bioremediation works on the field, screening for indigenous microbes is the norm since these microbes would have an optimum temperature close to the temperature of the site chosen for bioremediation. Fig. 4 shows the optimum temperature for growth of Isolate DRYJ7 was at 15°C. A dramatic drop in growth was seen at temperatures lower or higher than this. Since bioremediation in Antarctica can only work in summer where the temperature hovers from 0 to 10°C, this isolate can still be used at this temperature range, however, at reduced capacity. Most of the commonly studied acrylamide/aliphatic amides-degrading bacteria are mesophiles with optimum temperature for growth in the range of 25 to 40°C. Ciskanik *et al.*, (1995) reported an optimum temperature for the growth of *Pseudomonas chlororaphis* at 26°C, whereas Prabu and Thatheyus (2007) reported 28°C as the optimum growth temperature for *Pseudomonas aeruginosa*. Kotlova *et al.* (1999), Nawaz *et al.* (1994) and Wang and Lee (2001) reported 30°C as the optimum temperature for the growth of *Rhodococcus rodochrous*, *Rhodococcus* sp. and *Pseudomonas stutzeri*, respectively. *Helicobacter pylori*, found in the human gut, had an optimum temperature of 37°C (Skouloubris *et al.*, 1997). In contrast, thermoactive bacteria need higher temperature for their growth. The

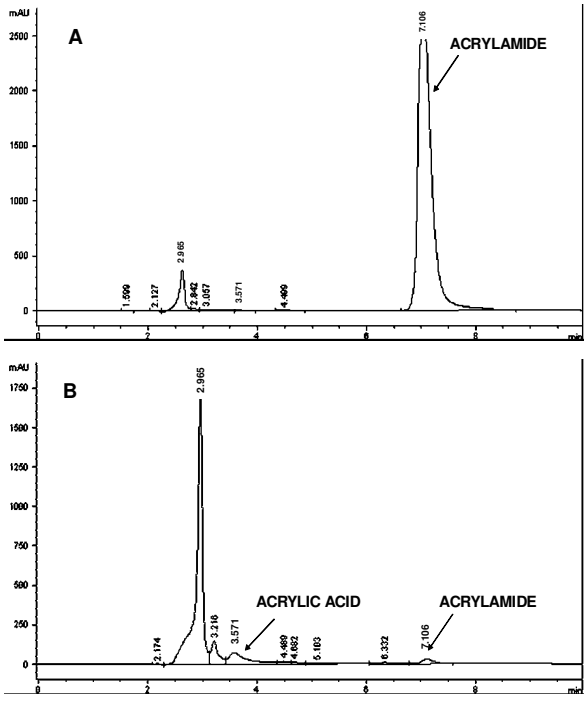

Fig. 7: Chromatogram of acrylamide degradation and in liquid culture at 0 day (A) and after 10 days of incubation (B). Acrylamide shows a retention time of 7.10 minutes whilst acrylic acid shows a retention time of 3.57 minutes

thermoactive *Pseudonocardia thermophilia* showed optimal growth at 50°C (Egorova *et al*., 2004) whereas *Brevibacillus borstelensis* BCS-1 needs 55°C (Baek *et al*., 2003).

Effect of acrylamide concentration: This experiment was carried out to determine the optimum concentration of acrylamide for the growth of Isolate DRYJ7. Different concentrations of acrylamide ranging from 100 to 2000 mgl^{-1} were used in this study. Fig. 5 shows that bacterial growth was increased as acrylamide concentrations were increased and reached an optimum at 500 mgl^{-1} acrylamide giving significantly higher cellular growth compared to 1000 mgl^{-1} ($p<0.05$). At higher concentrations of acrylamide, bacterial growth dramatically decreased. This is probably due to the inhibitory effect of acrylamide on thiol groups of proteins (Cavins and Friedman., 1968).

The optimum growth at 500 mgl^{-1} is similar to the optimum acrylamide concentration reported for the growth of *Pseudomonas stutzeri* at 440 mgl^{-1} (Wang and Lee, 2001). At 500 mgl^{-1}, bacterial growth showed a 2-day lag phase before degradation took place concomitant with an increase in cellular growth. Cellular growth and acrylamide degradation reaches a plateau on the 4th day onwards with only 30% of acrylamide being degraded at this point (Fig. 6). Acrylic acid as a metabolite was detected in the media using the same conditions of HPLC as for acrylamide (Fig. 7). Acrylic acid was also the intermediate detected in the degradation media of *Pseudomonas stutzeri* grown in 440 mgl^{-1} acrylamide under aerobic conditions (Wang and Lee, 2001). Nawaz *et al.*, (1998) and Prabu and Thatheyus (2007) studied the comparison of the degradation of acrylamide by immobilized and free cells of *Rhodococcus sp*. and *Pseudomonas aeruginosa,* respectively, indicating that immobilized cells are faster than free cells in degrading acrylamide to acrylic acid and ammonia. The rapid transformation of acrylamide to acrylic acid and ammonia is due to the fact that immobilized cells contain a high concentration of cells at their peak catabolic activity whereas free cells take time to multiply. Immobilization of this bacterium would be carried out in the future to increase acrylamide degradation capacity.

In conclusion, we have isolated and characterized an acrylamide-degrading bacterium from Antarctic soil. Our works sought to increase the repertoire of bacterial species able to degrade acrylamide. Knowledge of the various optimized parameters would facilitate an easy and more effective translation of the laboratory results to the fields. We have studied the effect of various parameters on acrylamide degradation from this bacterium. From the present study, all of these parameters play a crucial role for the bacterial growth and acrylamide degradation. Understanding acrylamide degradation is vital not only in the microbiological point of view, but the prospect of lowering acrylamide concentrations via bioremediation can lower the potential of acrylamide presenting itself as a pollutant and contaminant. This is the first report of an acrylamide degrading microbe from the Antarctic. Despite of no report on acrylamide pollution in Antarctica, the variety of catabolic properties of microbe is such that it has been reported that soil obtained from area far from human impact showed a similar proportion of hydrocarbon degrading bacteria compared with those found in pristine soils Although it is commonly known that the biodegradation potential of strains isolated from contaminated environment were higher than those originating from non-contaminated biotopes, Riis *et al*., (1995) and Chaîneau *et al*., (1999) reported that strains isolated from the control plot were as active as those selected in the treated plots. Currently, work is underway to purify the amidase from this bacterium.

Acknowledgments

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