Isolation and characterization of an acrylamide-degrading Antarctic bacterium


1Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
2Faculty of Animal Husbandry, Andalas University, Padang, Indonesia
3Instituto Antartico Argentino, Centrio 1248 (1010), Buenos Aires, Argentina

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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 DRY7 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 mg l⁻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 polyacrylamide 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 Antarctica.

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 mg l⁻¹ acrylamide as the sole nitrogen source. The growth and maintenance of the isolate was carried out according to the modified medium of Giskanik et al. (1995). Briefly, the medium contained (per liter), 10 g of glucose, 0.5 g of MgSO₄·7H₂O, 0.05 g of FeSO₄·2H₂O, 100 mg l⁻¹ acrylamide and 1 ml of the following trace elements; ZnSO₄·7H₂O, 0.34 μM; MnCl₂·4H₂O, 0.15 μM; H₂BO₃,
4.85 μM; CoCl$_2$·6H$_2$O, 0.84 μM; CuCl$_2$·2H$_2$O, 0.05 μM; NiCl$_2$·6H$_2$O, 0.08 μM and Na$_2$MoO$_4$·2H$_2$O, 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 mg/l 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 polycarbonate filter) 20 µl sample was injected into the HPLC system. The injection volume was 20 µl. The column used was a Shodex GPC-1020 column (Showa Denko K.K., Tokyo, Japan). The eluent was 0.02 M phosphate buffer (pH 7.0) with a flow rate of 1 ml/min. The absorbance was monitored at 220 nm.

For the determination of acrylamide in the supernatant, a portion of the supernatant was filtered using a 0.45 µm polycarbonate filter to separate cells from the supernatant. The filtered 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 Bioline T Gradient PCR (Bioline, London, UK). The PCR mixture contained 0.5 μM 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 Primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-AAGGAGGTGATCCAGCCGCA-3’) corresponding to the forward and reverse primers of 16S rDNA respectively (Devereux and Wilkinson, 2004). PCR was performed using 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 16S rDNA of Pseudomonas putida (Shukor et al., 2007), Pseudomonas stutzeri (Alt and Kirsch; 1975), 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 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 to be Gram-negative rod, is oxidase and catalase positive. The sequences were compared with the GenBank database using the Blast server at NCBI (Altschul 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 (Ratnesh, 2000), with Bacillus subtilis as the outgroup in the cladogram. Evolutionary distances 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 M1 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 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 place strain DRYJ7 into 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
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(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 rodchrous (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. 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.

Bacterial species used in this study include Bacillus subtilis strain KT1003 [AB115959], Pseudomonas sp. SN1-1 [AJ419675], Pseudomonas veronii [DQ392980], Pseudomonas sp. KBS04 [AY653221], Pseudomonas marginalis strain NZC8 [AF364098], Gamma proteobacterium BT-P-1 [AY398822], Pseudomonas sp. BWDD-24 [DQ219370], Pseudomonas veronii strain AIXdTBTE [AY512618], Pseudomonas veronii strain AIXB3-5 [AY512619], Pseudomonas sp. 'A1Y dBTEX1-5' [AY512643], Pseudomonas sp. 'ARDRA PS1' [AY364085], Pseudomonas sp. PM-2001 [AF321239], Pseudomonas sp. K94.08 [AY456703], Pseudomonas sp. ST5 [AF396931], Pseudomonas sp. 4N1-3 [AJ419672], Pseudomonas sp. 4N4-1 [AJ419673], Strain Dr.YJ7, Pseudomonas grumontii [AF268029], Pseudomonas brenneri [AF268968], Pseudomonas sp. A2 [AF195875], Pseudomonas sp. KA2 [AF195876], and Pseudomonas sp. HHS12 [AJ846278].

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.
to 2% glucose appears to be the optimum (Nawaz et al., 1995) when acrylamide is used as a nitrogen source. In several cases, degradation have also identified glucose as the best carbon source in general (King et al., 1992). Previous studies in acrylamide-starch have 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 6 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 robothrous*, *Rhodococcus* sp. and *Pseudomonas stutzeri*, respectively. *Helicobacter pylori*, found in the human gut, had an optimum temperature of 37°C (Skouloubiris et al., 1997). In contrast, thermoactive bacteria need higher temperature for their growth. The
and Thateyus (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 microbial 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.

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References


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