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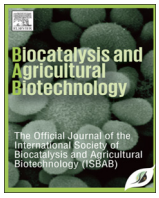

A novel nitrile-degrading enzyme (nitrile hydratase) from *Ralstonia* sp.ZA96 isolated from oil-contaminated soils

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ABSTRACT

In this study, a bacterial strain with capable of degrading nitrile compounds was isolated from oil-contaminated soils. Aacrylonitrile removal by the bacterium was analyzed using gas chromatography-mass spectrometry (GC-MS). The isolate was identified as Ralstonia sp.ZA96 by biochemical and molecular analyses. Purification of nitrile hydratase from the strain was performed using Q-Sepharose chromatography. The results showed that the 23 kDa-purified enzyme had an optimal activity at pH 8.5 and 25 °C. $Zn^{2}+$ ion (at 10 mM) and $Ca^{2,}$ ion (at 2 mM) had a positive and negative effect on the enzyme activity, respectively. Ethylenediaminetetraacetic acid (EDTA) caused a significant reduction in the enzyme activity. The activity in the presence of 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and dithiothreitol (DTT) was almost maintained, whereas SDS at 10 mM reduced 43% of the enzyme activity. Dimethyl sulfoxide (DMSO) and allyl alcohol had a positive effect on enzyme activity, while chloroform decreased the enzyme activity. The studied enzyme showed a high specificity to aliphatic nitriles including potassium ferrocyanide, potassium hexacyanoferrate, and ammonium thiocyanate. Our results suggest that this nitrile-degrading enzyme had potential to be utilized as a novel candidate for industrial applications.

1. Introduction

Cyanides are among the nitrile compounds that are highly toxic to living organisms exerting their toxicity through the inactivation of the respiratory system (Leavesley et al., 2008).Nitriles are among the organic compounds that are increasingly being produced in various natural and synthetic forms (Víctor et al., 2018). Cyano-glycosides, which are secondary plant metabolism products, rapidly decompose through plant tissue damage into products such as sugars; ketone compounds, aldehydes, as well as a highly toxic compound namely hydrogen cyanide (Gleadow and Møller, 2014).Also, nitrite compounds are widely used in the form of various industrial products such as solvents, plastics, synthetic rubber, herbicides and drugs (Banergee et al., 2002).In addition to urban and agricultural wastewaters, manipulation of metals, mining, etc. are among the industries whose output of wastewater contains nitrile compounds (Fang et al., 2015; Jung-Min Choi et al., 2015).

By acting on cellular DNA, these compounds produce carcinogenic changes in human, also environmental pollution might take place due to their long half-life and washing with rain and flooding into farmlands that use surface water (Horton et al., 2002). Various methods have been used to remove cyanide from contaminated wastewater, including the removal of chemical by hydrogen peroxide (Baxter and Cummings, 2006; Rahul et al., 2017; Ezzi and Lynch, 2005), sulfur dioxide and alkaline chloride (Demopoulos and Cheng, 2004). Nitrile bond in nitrite compounds is very stable, resulting in the necessity of acidic (6 M acid chloride) or alkali (2 M sodium hydroxide) conditions and high temperature for their chemical hydrolysis. In addition, the production of side products such as toxic cyanide acid and high amounts of salt can cause many problems. Due to these disadvantages, microbial-enzymatic degradation with advantages such as physiological and physicochemical control, high enzymatic diversity and amounts of enzyme production can be an effective way to remove high-toxic nitriles from the environment or to convert them into less-toxic compounds (Cantarella et al., 2006).The enzymatic hydrolysis of the nitriles is accomplished by two different enzymatic pathways.

In the first pathway, a nitrilase (E.C.3.5.5.1) catalyzes the direct hydrolysis of the nitriles into the corresponding acids (Banergee et al., 2002). In the other pathway, nitrile hydratase (E.C.4.2.1.84) converts nitriles into their corresponding amides (Brady et al., 2004).

Nitrile decomposition activity is rarely seen in nature. Low enzyme activity is observed in only three families of 21 examined plant families and a limited number of fungal species. This activity is more prevalent in bacteria. Some of them, such as *Acinetobacter*, *Rhodococcus, Klebsiella*,

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Pseudomonas, *Arthrobacter*, *Corynebacterium* and *Nocardia* use nitriles as nitrogen and carbon sources (Rapheeha et al., 2016).The nitrile hydratease enzyme in bacteria is commonly expressed simultaneously with an amidase that converts amide produced by releasing ammonia into an acid. The enzyme that was identified for the first time as a part of the metabolic pathway of nitrile decomposition in germs in the production of nicotinamide and 5- cyano-valeramide, is more useful in the production of pharmaceutical intermediates, agriculture, and acrylamide production (Chuchat et al., 2016).The nitrile hydratases have two structural features: Nitrile hydrates that contain iron ion (Fe) at their active site and enzymes that contain cobalt (Co) ions at their active site (Prasad et al., 2004). This enzyme is intracellular, so its purification requires the breakdown of bacterial wall. The enzyme has two subunits α and β that show molecular weights between 22 kDa and 29 kDa, with the metal ions located between two subunits (Prasad et al., 2009).

The optimum pH of the nitrile hydratese enzyme is between 6.5 and 8.5. Also, the optimum temperature ranges from 20 to 35 °C and very few of them can withstand at high temperatures such as 40, 50 and 60 °C (Prepechalova et al., 2001).The substrate of this enzyme can be aliphatic or aromatic type nitrile. The obtained enzymes from different bacteria showed different effects on substrates. Acrylonitrile as a primary material is widely used in industrial and commercial processes. Biodegradation of acrylonitrile in industrial wastewater has been reported by some researchers using various bacterial strains including *Arthrobacter*, *Corynebacterium*, *Nocardia* and *Pseudomonas*, *Rhodococcus*, *Acinetobacter* and *Citrobacter* (Wang et al., 2004). In other studies, some of these strains have been successfully used to remove acrylonitrile from soil (Lovasoa et al., 2017).

In current study, our objective was to isolate and characterize nitrile hydratase from bacterial flora of oil-contaminated soils that was able to degrade both aliphatic and aromatic nitriles.

2. Materials and methods

2.1. Chemicals

The chemicals including acetonitrile, acrylonitrile, malonitrile, sodium cyanide, potassium thiocyanide, potassium cyanide, potassium ferrocyanide, potassium hexacyanoferrate, 4-bromobenzonitrile, 2-(bromomethyl) benzonitrile and ammonium thiocyanate and bacterial culture media were purchased from Ioba Chemie and Himeda (Mumbai, India). Solvents, detergents, inhibitors and other chemical compounds were of analytical grade.

2.2. Isolation of bacteria from oil-contaminated soils

The soil samples were taken from the soils around the oil reservoirs and were completely mixed with deionized water in a ratio of 1–9 after transferring to the laboratory. After sedimenting the soil at the bottom of the tube, the surface liquid was transferred to another test tube. The dilution was carried out in three stages with a ratio of 1–9. The final diluent was then poured onto the surface of the prepared nutrient agar medium and spread on the surface of the medium followed by 24–48 h incubation at 37 °C. Then each of the grown colonies was purified separately on the new culture medium.

2.3. Culturing on a special culture medium contains nitrile

Acrylonitrile removal ability of each purified colony in specific culture medium was evaluated through gas chromatography-mass spectrometry (GC-MS). The specific culture medium components as follow (g/l): glucose 10, K_2HPO_4 0.5, $MgSO_4$. 7 H_2O 0.5, KH_2PO_4 0.5, $CoCl_2$ 0.01, $FeSO_4$ 7 H_2O 0.01, yeast extract 0.2 (pH 7.2–7.5). Autoclave was performed at 121 °C for 15 min. After cooling the medium, 0.2% (V/V) acrylonitrile was added to the specific culture medium. Then, the previously incubated bacteria for 24 h at 37 °C in nutrient broth, 10% (v/v) were inoculated into the above mentioned medium followed by incubation in a shaker incubator at 25 ± 5 °C and 150 rpm for 3–4 days.

2.4. Checking the amount of acrylonitrile removal through GC

The residual amount of acrylonitrile in specific culture medium was evaluated before and after incubation for each purified bacteria by GC (model CP 3800) with a mass detector Saturn 2200 (Varian, USA). The separation was achieved on a capillary column (30 m × 0.25 mm × 0.25 μm) using the program: 35 °C for 3 min; a rate of 13 °C/min to 85 °C and 25 °C/min to 225 °C.

2.5. Extraction of cell extract

Cell extract was prepared from the bacterium grown in specific culture medium containing acrylonitrile (2% v/v) with the maximum ability of removing acrylonitrile from the medium. Stages of cell extract preparation:

Stage 1. centrifugation: To isolate the bacteria from the culture medium, centrifugation was performed at 10,000 rpm for 20 min at 4 °C.
Stage 2: precipitate washing: to do this, the supernatant was discarded and, sodium phosphate buffer (20 mM with pH 7.8) was added and centrifuged as the first stage.
Stage 3: sonication: the supernatant was discarded and a suspension of 20 ml of bacteria was prepared using snicate suspension solution (18 ml of sodium phosphate buffer, 1 ml of 5% glycerol, 1 ml of 10 mM PMSF). A suspension of 20 ml of bacteria was disrupted using a sonicator (Misonix-S-4000 Ultrasonic Liquid Processor) at a nominal power of 70 W for 20 s periods; each period of disruption was set to 20 s ON and 20 s OFF at 4 °C up to 20 periods.
Stage 4. centrifugation: the centrifugation of the solution of bacterial suspension was carried out at 12,000 rpm for 20 min at 4 °C. The supernatant was used as a cell extract for purification of the enzyme.

2.6. Enzyme purification

After the above steps, the cell extract was inserted into the dialysis bag. Then, the dialysis bag containing the extract was stored in a sodium phosphate buffer 20 mM with pH 7.8)buffer A)for 24 h at 4 °C. The dialyzed extract was loaded onto an anion exchange Q-Sepharose column. With a peristaltic pump at a flow rate of 1.2 ml/min different fractions of 2.5 ml volume were collected with a gradient of sodium chloride (0–1 M NaCl in buffer A). Finally, the protein content and enzyme activity in each fraction were estimated.

2.7. Enzyme activity

In order to assay the enzyme activity 140 μL of sodium phosphate buffer (50 mM and pH 7.2), 100 μl of acrylonitrile (200 mM) and 60 μL of enzyme solution were mixed and incubated at 29 °C for 30 min. By adding 1 M HCl (70 μL) to the mixture, the reaction was stopped and the rate of acrylamide production was detected at wavelength of 220 nm. Protein concentration was estimated using the Bradford method. An unit activity was defined is an amount of enzyme producing 1 μmol amide from nitrile per minute. An extinction coefficient of 2660 M^{-1}cm was used to determine acrylamide concentration (Liya et al., 2007).

2.8. SDS PAGE

This technique was used to confirm the purity and the molecular

mass determination of the enzyme. A separating gel 15%(w/v), stacking gel 7%(w/v), cathode buffer (Tris 100 mM, SDS1%, glycine 100 mM, pH 8.2) and anode buffer (Tris 200 mM, pH 8.9) were used. Commissio Blue R250 (1%) was utilized to detect protein bands. The molecular mass of the enzyme was estimated using a Vivantis protein ladder (California USA).

2.9. DNA extraction and proliferation of 16S rRNA

DNA extraction was performed by DNA extraction kit (Sinagen, Iran). The universal primers including of 27f (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1492R (5′- TACGGCTACCTTGTTACGACTT-3′) were used for amplification. The PCR reaction was conducted in 25 µl volume with the following program: 2 min at 94 °C, 28 cycles of 2 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, plus an additional 10-min cycle at 72 °C. Then, 5 µl of the PCR product and the 1 kb size marker were electrophoresed on 1% agarose gel. The PCR product was purified by a PCR purification kit (Bioneer, South Korea) and sequenced by Macrogen Company (Seoul, South Korea).

2.10. Enzyme characterization

2.10.1. Effect of pH and temperature

To determine the optimal pH, enzyme activity was measured at different pHs (5–10). Two types of buffer were used, including sodium phosphate buffer 20 mM (pH 5–8) and Tris-HCl buffer 20 mM (pH 8–10). Using different buffers, different pHs were prepared and for each pH, a mixture of buffer, substrate and enzyme was prepared according to the enzyme assay and the enzymatic activity was calculated. To determine the optimum temperature, the thermal range between 30 and 80 °C with an interval of 5 °C was used. To determine thermal stability of the enzyme, the purified enzyme was incubated (without substrate) at 50, 60 and 70 °C for 75 min and then the sample tube was cooled. The residual enzyme activity was determined according to the enzyme assay.

2.10.2. Effect of metal ions, inhibitors and organic solvents

The effect of different ions on the enzymatic activity was investigated. To measure the enzyme activity, sodium phosphate buffer 20 mM with pH 8.5 and 2, 10 mM concentrations of different ions including Fe^{2+},Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+} were used. Enzyme activity was measured and compared with the control. The effect of detergents on the activity of the enzyme was investigated by using sodium phosphate buffer (20 mM, pH 8.5) and concentrations of 1 and 10 mM of SDS (sodium dodecyl sulfate), cetyl trimethylammonium bromide (CTAB), Triton X-100, Tween-80, Naclo and H_2O_2. The enzyme activity was determined as abovementioned (section 2-7). Additionally, the effect of inhibitors on the enzyme activity was investigated. Enzyme activity was determined in the presence of various inhibitors at concentrations of 2 and 10 mM. These inhibitors include: ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), and sodium azide (NaN_3). The effect of different solvents was also studied. First, different solvents including propanol, chloroform, acetone, allyl alcohol, toluene, dimethyl sulfoxide (DMSO) and benzene were prepared at concentrations of 2 and 10 mM. Then, the residual nitrile hydratase activity was determined and compared with non-solvent control samples.

2.10.3. Substrate specificity and kinetic parameters of the enzyme

The substrate specificity of the nitrile hydratase enzyme purified investigated in the presence of various types of aliphatic and aromatic nitriles, such as acrylonitrile, acetonitrile, malonitrile, sodium cyanide, potassium cyanide 4-bromobenzonitrile, 2-(bromomethyl) benzonitrile and ammonium thiocyanateas substrates. The enzyme activity was examined in the presence of substrates under standard conditions. The enzyme activity in the presence of acrylonitrile substrate was considered as an indicator. Determination of kinetic parameters, including Michaelis-Menten constant (K_m) and the maximum reaction velocity (V_{max}) for the purified nitrile hydratase using the different concentrations of acrylonitrile (50–300 mM) was performed. The GraPhpad prism 6 software was used to plot the Michaelis-Menten graph, then, by reversing the data for each axis, the Lineweaver–Burk plot was drawn and on the base of this plot, the kinetic parameters of K_m and V_{max} were estimated.

3. Results

3.1. Screening of bacteria with acrylonitrile degradation ability

After separation and purification colonies from the soil samples, each purified colony was grown in specific medium containing acrylonitrile at 29 °C for 72 h. The amount of acrylonitrile in a specific culture medium was determined before and after incubation by gas chromatography (GC). A strain that showed the highest acrylonitrile removal activity from a specific culture medium was selected and used for the next steps. Chromatograms obtained from different strains showed that the desired bacteria have the highest ability to remove acrylonitrile from the specific culture medium (Fig. 1). Results showed that in the presence of acrylonitrile substrate, the desired bacteria were able to grow after 72 h and used acrylonitrile as a source of carbon and nitrogen and thereby removing it from the medium. Therefore, owing to the high ability of the isolated strain to produce nitrile hydratase into the specific medium it was selected for further study.

3.2. Identification of the selected strain

The PCR amplification of 16S rRNA gene showed a band on 1% agarose gel with length of about 1500 bp (Fig. 2A). Sequencing of the PCR product was accomplished by Macrogen Company. Multiple alignment of the sequenced gene along with other 16S rRNA sequences in the NCBI Genbank showed that the isolated bacterium belongs to the genus *Proteobacteria* and has 94% and 87% similarity to *Ralstonia* sp.WTW02 and *Ralstonia syzygii subsp. celebesensis* UQRS 481, respectively. In addition, this sequence has phylogenetic relationship with *Ralstonia* sp.AJ11, *Ralstonia* sp. B8, *Ralstonia* sp. MB80. Based on the results of the phylogenetic tree, the strain was named as *Ralstonia* sp.ZA96 (Fig. 2B). Several other bacteria of the genus *Proteobacteria* identified which are able to degrade some chemical compounds; for example, *Ralstonia* sp. MRL-TL isolated from hot springs which was able to degrade poly ε-caprolactone compound by an extracellular degrading enzyme as PCL depolymerases (Shah et al., 2015) and also *Ralstonia* sp. PS12 that contained the dichlorotoluene degrading enzyme(Pollmann et al., 2002). In another study, two thermophilic bacteria, *Ralstonia solanacearum* and *Ralstonia eutropha* H16, were identified that have extracellular lipase enzymes that are able to degrade some lipid compounds (Moayad et al., 2018; Lu et al., 2013).)

3.3. Enzyme production

After examining the different strains isolated from oil-contaminated soil samples and checking the results of gas chromatography (GC), the isolate of *Ralstonia* sp.ZA96 showed nitrile hydratase activity. Our results showed that optimal temperature of growth bacterium (OD_{600}) was at 29 °C and the maximum enzyme production was observed after 84 h of incubation. The log phase began after 48 h and reached to stationary phase after 72 h. Generally, the highest enzyme production was in the stationary phase, because the growth-supporting sources in the specific culture medium (glucose and yeast extract) supplemented by acrylonitrile are consumed as a source of carbon and nitrogen in log and stationary phases.

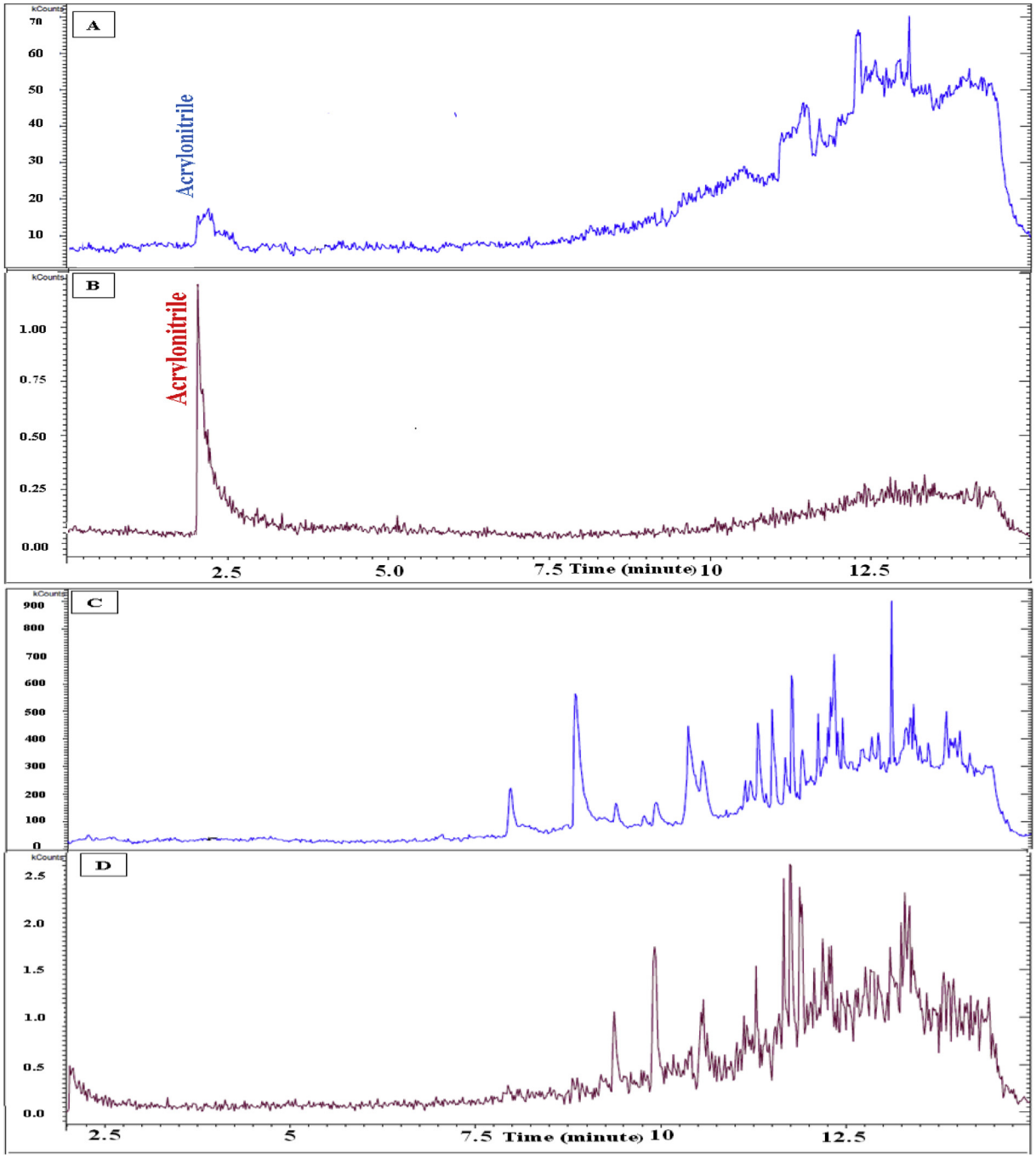


Fig. 1. Gas chromatography)GC) of a specific culture medium containing acrylonitrile at the onset and the end of incubation with the isolated strain. A: Chromatogram of specific culture medium containing acrylonitrile (20 µl ml^{-1}) before incubation. B: Chromatogram of specific culture medium containing acrylonitrile before incubation in M/Z 53 (molecular mass of acrylonitrile (with retention time 2 min. **C**: Chromatogram of specific culture medium containing acrylonitrile after incubation (at 29 °C for 72 h). D: Chromatogram of specific culture medium containing acrylonitrile after incubation in M/Z 53 with retention time of A2 minutes.

3.4. Enzyme purification

Due to the fact that the nitrile hydratase is intracellular, the cell wall of the bacterium was broken down by sonication. According to OD_{280} and the enzyme activity of each fraction, the most active fraction was selected. Results revealed that the maximum enzyme activity was observed in the fraction of 15 and eluted at 0.4 M NaCl in buffer A (Fig. 3A).

The purified enzyme was achieved by a 20.3-fold increase in the specific activity and a yield of 66.4% as it compared to the crude enzyme (Table 1).

3.5. Enzyme characterization

3.5.1. Molecular mass

The molecular weight of the purified nitrile hydratase units was determined by SDS-PAGE with 15% and 7% gel. SDS–PAGE results displayed that both subunits of the enzyme was the same molecular weight of 23 kDa for the nitrile hydratase obtained from *Ralstonia* sp.ZA96 (Fig. 3B**).**

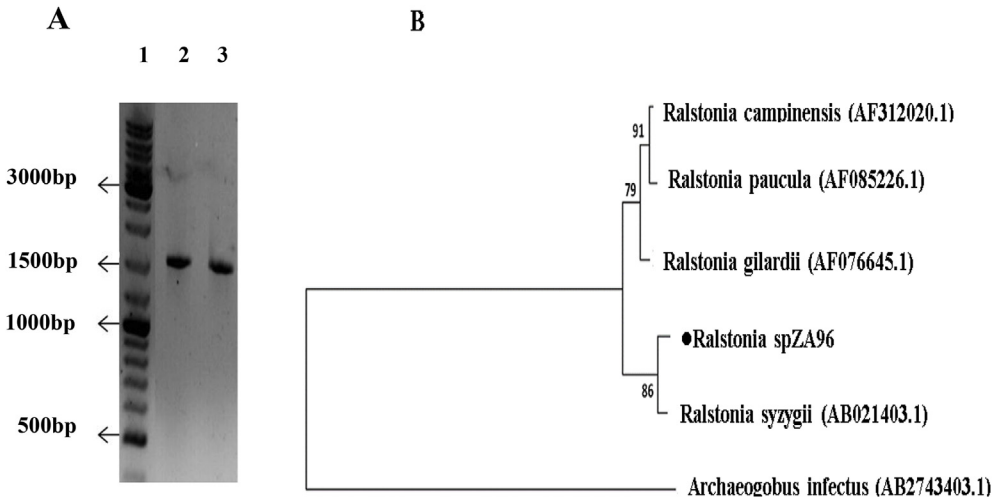


Fig. 2. The phylogenetic tree of 16S rRNA sequences of *Ralstonia* strains and results of PCR. **(A)** Agarose gel electrophoresis of 16S rRNA gene amplification by PCR. Lane 1, standard marker. Lanes 2 and 3, PCR products of 16S rRNA gene of the selected strain. (**B**) 16S rRNA sequences of the corresponding strains obtained from GenBank and Neighbor-joining cladogram was constructed using MEGA7. The isolated strain was represented by *Ralstonia* sp.ZA96. Accession number of the each sequence had been typed at the end of its corresponding branch. The sequence of AB274307 (*Archaeogobus infectus*) was used as an outgroup.

3.5.2. Temperature and pH profiles

The effect of pH on the purified enzyme activity was performed to determine the optimal pH. The enzyme activity was determined in the pH range of 5–10. The results showed that the enzyme had the highest activity at pH 8.5 (Fig. 4A). Noteworthy, this enzyme was active in a wide range of pHs so that the enzyme activity maintained at pH 5 and pH 9.5 up to 85% and 75%, respectively. The effect of temperature on the enzyme activity was assayed from 10 to 60 °C. The *Ralstonia* sp ZA96 nitrile hydratase was active in a range of temperatures (20–50 °C) with an optimum temperature of 25 °C. The enzyme activity decreased at 50 °C and 60 °C to 31% and 41%, respectively (Fig. 4B).

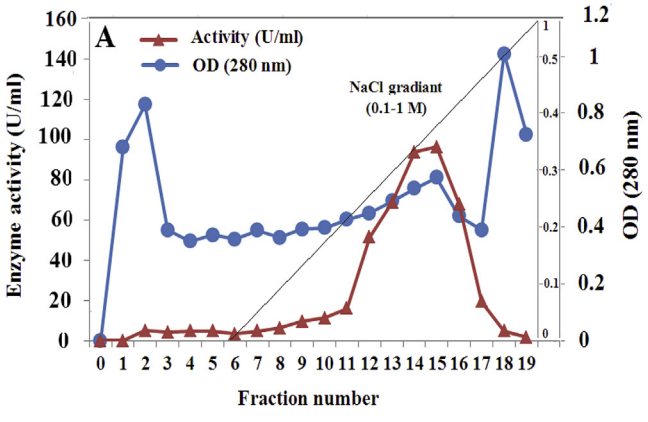
3.5.3. The effect of metal ions on the nitrile hydratase activity

The effect of monovalent and bivalent ions on enzyme activity was evaluated. The remaining enzyme activity was determined for each ion at concentrations of 2 and 10 mM and compared with control sample (Fig. 5A). The results showed that some ions, including Zn^{2+} had a positive effect on the enzyme activity at a concentration of 10 mM. In addition, in the presence of Fe^{+2}a high amount of enzyme activity was maintained at both concentrations.

The results also showed that Ca^{2+}at 2 mM the highest reduction in the enzyme activity compared to other ions. This can be due to the effect of Ca^{2+} ions on the structural ion in the enzyme (possibly Fe^{2+}) which changes the properties of the active site of the enzyme. In general, the average amount of remaining activity at 2 and 10 mM of studied ions showed that the nitrile hydratase activity of *Ralstonia* sp.ZA96 retains in the presence of all studied ions by more than 75%.

3.5.4. The effect of detergents and inhibitors on the activity of the enzyme

The effect of inhibitors on the enzyme activity was investigated at concentrations of 2 and 10 mM (Fig. 5B). The results showed that EDTA had the most reduction on the enzyme activity at a concentration of 2 mM. EDTA is a chelating agent of metal ions (such as Ca^{2+} and Fe^{+2}), it can form a complex with the metal ion in the structure of nitrile hydratase leading to decrease the enzyme activity. Therefore, according to available evidence, the nitrile hydratase of *Ralstonia* sp.ZA96 is probably an enzyme containing Fe^{+2} ion)*Fe*-type(. The results also showed that the two compounds of DTNB and DTT at 2 and 10 mM had the least effect on enzyme activity. Since DTNB and DTT effect on the sulfhydryl group of cysteine residues, it could be concluded that cysteine in the structure of the studied nitrile hydratase, is less accessible to environmental agents. The results obtained from the effect of detergents on the enzyme activity are presented in Fig. 5C. The results showed that SDS at concentration of 10 mM reduced the enzyme activity by 43%, which shows the greatest effect on the enzyme activity compared to other detergents. This can be due to the fact that the enzyme studied contains hydrophobic amino acids in the areas available for SDS that it interacts to these areas and the enzyme net charge changes to negative charge resulting in disruption of the equilibrium of the enzyme charge and decreases the enzyme activity. CTAB (cationic detergent) showed a lower effect on the enzyme activity than SDS. Among the detergents used, Triton X-100 had the least effect on enzyme activity. Also, H_2O_2, as a potent oxidizing protein, can reduce disulfide bond in the enzyme structure, had a lower effect on the enzyme activity, which indicates the low number of disulfide bonds or



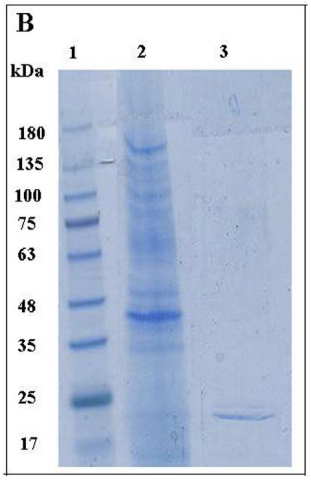


Fig. 3. Anion-exchange chromatography on Q-Sepharose column (3 × 10 cm) and SDS-PAGE analysis. (**A**) Different fractions were collected with a gradient of sodium chloride (0–1 M NaCl in sodium phosphate buffer 20 mM with pH 7.8). The enzyme was eluted at 0.4 M NaCl in fraction 14 and 15. (**B**) SDS-PAGE analysis of the purified nitrile hydratase. Lane 1: Protein Marker (11–180 kDa), Lane 2: crude extract, Lane 3: the purified enzyme by Q-Sepharose chromatography (fraction 15).

Table 1
Purification steps of the nitrile hydratase enzyme isolated from *Ralstonia* sp.*ZA96*.

Purification step	Volume (ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total protein (mg)	Total activity (U)	Fold	Yield (%)
Sample prior to sonication	20	2.75	6.9	55.0	379.5	1.0	100
After dialysis	18	1.13	13.8	20.5	282.9	2.0	74.5
Q-Sepharose	2.5	0.72	140.0	1.8	252.0	20.3	66.4

unavailability of these bonds to H_2O_2 in the enzyme's structure. This is supported by the low of the effect of DTNB and DTT agents on the enzyme activity.

3.5.5. The effect of organic solvents on the enzyme activity

The presence of organic solvents creates new properties in the structure of the enzymes that are never seen in aqueous environments (Simon et al., 2007). In this study, the effect of organic solvents on nitrile hydratase activity showed that DMSO and allyl alcohol at concentration had a positive effect on the enzyme activity. Furthermore, the enzyme activity was largely maintained in the presence of toluene, acetone and benzene. While, the enzyme activity in the presence of chloroform decreased in comparison to the rest of organic solvents (Fig. 5D).

3.5.6. Substrate specificity and kinetic parameter

Substrate specificity of the nitrile hydratase isolated from *Ralstonia* sp.ZA96 was determined using two types of aliphatic and aromatic substrate as shown in Table 2. Acrylonitrile conversion into acrylamide was considered as an index of 100% and the conversion of the rest of the nitriles into corresponding amides was compared with it. The purified enzyme had a high specificity to the aliphatic substrate containing potassium and iron including potassium ferrocyanide and potassium hexacyanoferrate, as well as ammonium thiocyanate compared with the rest of the substrate used in this study. Moreover, the purified enzyme affected on the aromatic substrates, so that, in the presence of 4-bromobenzonitrile, the enzyme showed an activity of 60%, but the least effect was seen on malononitrile.

The values of kinetic parameters were estimated for acrylonitrile as substrate by Lineweaver–Burk plot. K_m and V_{max} values were found to be 29.63 mM and 4.038 μmol min^{-1} mg^{-1}, respectively.

4. Discussion

Nitrile degrading bacteria can play an important role in removal of nitrite compounds from the water and soil environments (Naveen et al., 2018; Ogbonnaya and Marie, 2014).Hence, bacteria that are able to withstand a higher tolerance range of pH, temperature, inhibitors, etc. can be more important in industrial applications. Among the bacteria, *Pseudomonases* are more important due to bearing plasmids that carry the gene for the use of oil compounds. These bacteria can degrade oil compound hydrocarbons with specific mechanisms (Das and Mukherjee, 2007). The bacterium studied is one strain of *Ralstonia* that is a genus of *Proteobacteria* (previously included in the genus *Pseudomonas*) (Garrity, 2001). In addition to *Proteobacteria*, other genera of bacteria have been isolated from oil contaminated soil samples such as *Acinetobacter, Kocuria* (Sebastián et al., 2014), *Serratia proteamaculans sp. Alcaligenes*, *Rhodococcus erythropolis* (Mingqian et al., 2017).

In this study, we isolated a bacterial strain from oil-contaminated soil with the ability to degrade both hydrocarbon and nitrile compounds to perform more effective in removing contamination from soil or water. *Ralstonia* sp.ZA96 had an optimal growth in nitrile containing medium and was able to remove it from the medium. The ability to remove acrylonitrile in other strains of bacteria including; *Rhodococcus sp. N774*, *Pseudomonas chlororaphis B23*, *Rhodococcus rhodochrous M33*, *Rhodococcus erythropolis* have been also observed (Bhalla et al., 2018; Ashwini et al., 2013). Herein, the nitrile hydratase of *Ralstonia* sp.ZA96 was a Fe-type nitrile hydratase contains two subunits α and β and both have a molecular mass of approximately 23 kDa with a high degree of amino acid sequence homology.

SDS-PAGE of *Ralstonia* sp.ZA96 of the purified enzyme showed that the enzyme has a band around 23 kDa. The similar results are found for a nitrile hydratase obtained from *Rhodococcus rhodochrous* PA-34 (Prasad et al., 2009), *Rhodococcus* sp. *AJ270* (Liya et al., 2007) and *Rhodococcus equi* A4 (Irena et al., 2001).

A series of nitrile hydratases has ferrous ion in their structures that is called Fe-type hydratase (Prasad et al., 2009; Villar-Acevedo et al., 2017). Reports show that Fe-type nitrile hydratase purified from different genera of bacteria have an ability to degrade aliphatic nitriles while show a limited effect on aromatic nitriles. For example, nitrile hydratase of *Pseudomonas chlororaphis B23* is a Fe-type nitrile hydratase unable to hydrate any benzonitrile or other aromatic compounds (Nagasawa et al., 1987). However, our study showed that Fe-type nitrile hydratase was able to degrade both aliphatic and aromatic nitriles.

Crystal structure analysis of Fe-type nitrile hydratase have showed that there are two coordinated cysteine with iron at the center of the active site(Villar-Acevedo et al., 2017; Martinez et al., 2015; Murakami et al., 2000). Therefore, cysteine is very important in enzyme-catalyzed reaction. Factors that affect the amino acid of the cysteine and sulfhydryl groups can inhibit enzyme activity such as sulfhydryl reagents and

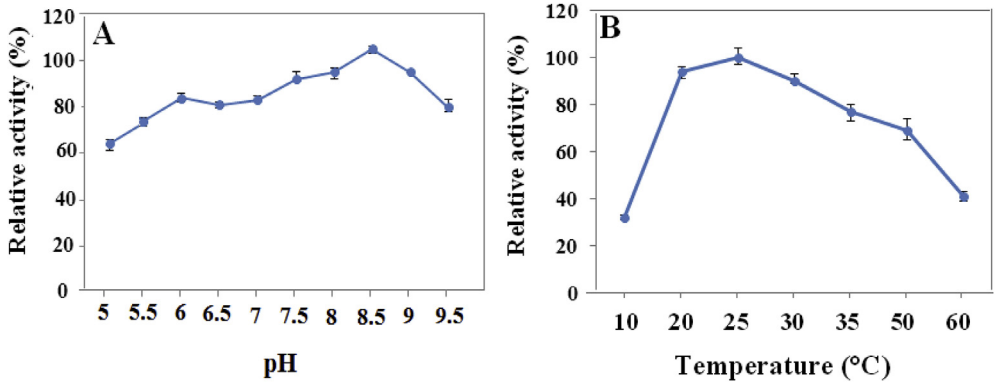


Fig. 4. Temperature and pH profiles of the purified enzyme. (**A**) Effect of pH on the activity of the nitrile hydratase. (**B**) pH- activity profile. The activities of the enzyme at pH 8.5 and temperature of 25 °C were taken as 100%.

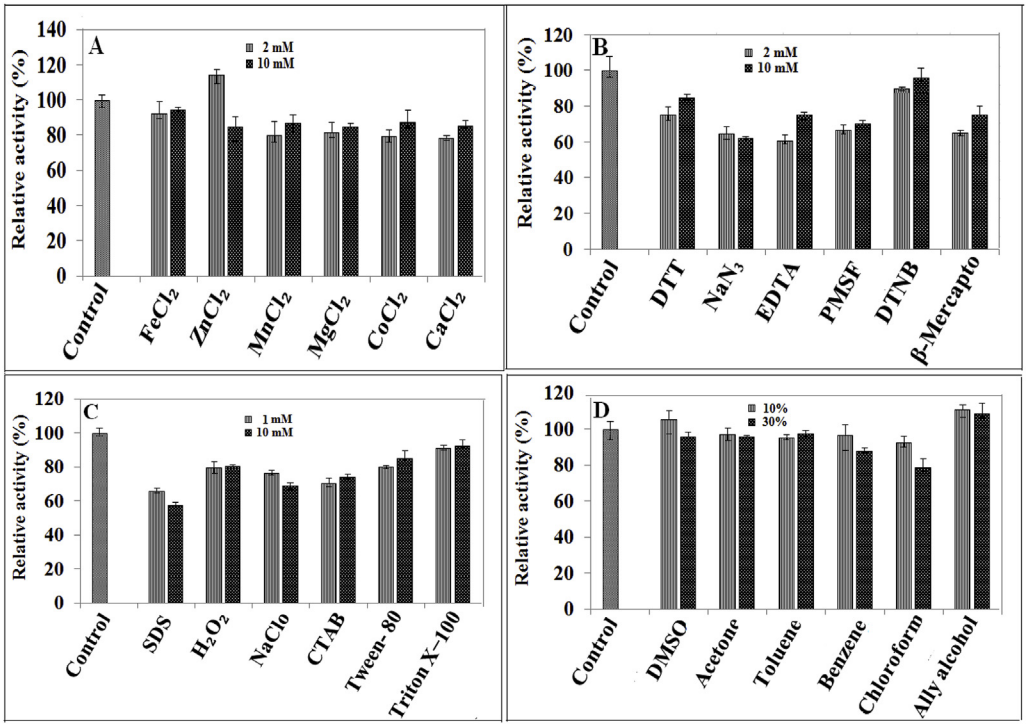


Fig. 5. The effect of different agents on *Ralstonia* sp.ZA96 nitrile hydratase activity. (**A**) Effect of various metal ions, (**B**) Effect of inhibitors, (**C**) Effect of different detergents, (**D**) Effect of organic solvents on the activity of nitrile hydratase.

Table 2
Substrate specificity of nitrile hydratase of *Ralstonia* sp.ZA96.

Organic solvent	Relative activity (%)
Acrylonitrile	100
Acetonitrile	19.4 ± 0.19
Malononitrile	10.4 ± 0.8
Sodium cyanide	25.4 ± 0.12
Potassium cyanate	42.6 ± 0.53
Potassium thiocyana	90.7 ± 0.54
4-bromobenzonitrile	60.0 ± 0.91
Potassium ferrocyanide	95.6 ± 1.5
Potassium hexacyanoferrate	64.4 ± 0.46
2-(bromomethyl)benzonitrile	38.3 ± 1.5
Ammonium thiocyanate	75.8 ± 0.15

Notes: Nitrile hydratase activity on acrylonitrile was considered as 100%.

oxidizing agents. For example, the activity of nitrile hydratase of *Rhodococcus* sp. *AJ270* was completely inhibited by potent oxidizing agents, such as H_2O_2[25]. Whereas, our results showed that the activity of nitrile hydratase was retained in the presence of sulfhydryl reagents of DTT, DTNB and oxidizing agent of H_2O_2. This difference is resulted from variations in either the 3-dimensional structure of the protein, or the post-translational modification between the two-nitrile hydratase of *Ralstonia* sp.ZA96 and *Rhodococcus* sp.*AJ270*.

Decreasing the activity of enzymes in the presence of some organic solvents can be due to tightening of the protein structure of the enzymes, denaturation and enzymatic inhibition. In fact, some organic solvents by interrupting water molecules around the protein can destroy three-dimensional structure (Miroliaei and Nemat-Gorgani, 2002). In general, the results showed that the purified nitrile hydratase could maintain 77% its own activity in all solvents. In application point of view, the nitrile hydratase enzyme can play an important role in the removal of nitrile compounds from the environment, the enzyme stability in various organic solvents is so important.

Our results also showed that nitrile hydratase of *Ralstonia* sp.ZA96 had a high activity in a wide range of metal ions, solvents, inhibitors and detergents. Therefore, it is a resistant enzyme against inhibiting agents compared to other nitrile hydratase and represents a good candidate for industrial use.

5. Conclusion

Ralstonia sp.ZA96 isolated from oil contaminated soil samples was able to remove acrylonitrile through the enzymatic activity of nitrile hydratase from its culture medium. Moreover, the study showed that this enzyme can be effective on a wide range of aliphatic and aromatic nitriles and has potential to reduce them from the environment. Therefore, due to biochemical features of the enzyme, it can be used effectively in different environments with different chemical conditions for degradation of nitriles.

Conflict of interest and ethical standards

Authors declare that they have no conflict of interest.

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