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BIODEGRADATION OF ANILINE BY *Enterobacter ludwigii* KH-A5 ISOLATED FROM THE SOIL AROUND SHIRAZ REFINERY, IRAN

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ABSTRACT

Aniline is a harmful substance that pollutes the environment and seriously endangers human health. In the present study five different bacteria were enriched and isolated from the soil around Shiraz refinery (Iran) as aniline degrading bacteria. They were identified as *Enterobacter ludwigii* KH-A5, *Raoultella planticola* KH-A2*, Alcaligenes faecalis* KH-A3*, Serratia marcescens* KH-A4 and *Microbacterium barkeri* KH-A1 based on 16S rDNA gene sequencing. The highest aniline degradation rate (96%) was observed by *E. ludwigii* KH-A5. This strain with the greatest amount of minimum inhibitory concentration (MIC) was the most aniline resistant bacterium. The optimum pH and temperature that supported biodegradation of aniline by strain KH-A5 were 7.0 and 28-35 °C, respectively. The maximum growth and biodegradation of aniline by KH-A5 were observed at the initial aniline concentration of 100 mg l^{-1}. The strain could growth on aniline up to concentration of 700 mg l^{-1} with the degradation rate of 11%. Further studies demonstrated that the addition of 0.5 g l^{-1} glucose or potassium nitrate as a second carbon or nitrogen source could slightly enhance the biodegradation efficiency from 96.0% to 99.0%. However, even more addition of glucose or potassium nitrate could not further enhance the biodegradation process but delayed the biodegradation of aniline by the strain KH-A5. It could be concluded that these new strains, particularly *E. ludwigii* KH-A5, have a potential use for bioremediation of the site contaminated with aniline.

Keywords: aniline, biodegradation, growth, *Enterobacter ludwigii* KH-A5, Shiraz refinery

1. Introduction

Aniline is a widely distributed environmental pollutant resulting from the manufacture of a number of products such as dyes, plastics, resins, pharmaceuticals, petro-chemicals and herbicides (Mohammed *et al.,* 2014; Yen *et al.,* 2008). Aniline has toxicity to life and plant and is considered as an important environmental hazard and is subject to legislative control by the European Economic Community (EEC) directive and in the Priority Pollutant List of US Environmental Protection Agency (Li *et al.,* 2010). This compound is known as the parent molecule in the large family of aromatic amines. Aniline dissolves slowly in water (approx. 35 g l^{-1} at 20 °C) but quickly dissolves in alcohol, benzene, and organic solvents (SCOEL/SUM/153 2010; Sihtmae *et al.,* 2010). It was revealed that the soil microorganisms while adaptation, to immediate environment developed certain metabolic pathways for effective utilization of synthetic compounds (Obinna *et al.,* 2008). Aniline is a major breakdown product of diphenylamine and p-aminoazobenzene (Zissi *et al.,* 1997). To treat wastewaters and affected soils

containing aniline and most of its derivatives, remedies often used are photodecomposition, auto-oxidation, electrolysis, resin adsorption or ozone oxidation but the cost of such treatments limits their application to small scale facilities (Ahmed *et al.,* 2010). Microbial degradation leading to different altered end products is thought to be the main mechanisms of toxic aniline elimination from the environment (Obinna *et al.,* 2008). Numbers of reports concerning the bacterial degradation of aniline had already been published, such as *Corynebacterium* sp. (Ahmed *et al.,* 2013), *Candida tropicalis* AN (Wang *et al.,* 2011), *Delftia* sp. (Liu *et al.,* 2002; Zhang *et al.,* 2008; Liang *et al.,* 2005) *Pseudomonas* sp. (Meyers 1999; Fukumori and Saint 1997; Bathe 2004) and *Acinetobacter* sp. (Takeo *et al.,* 1998; Fujii *et al.,* 1997). Li *et al.* (2007) isolated strain PN1001, which is a member of the *Pseudomonas* species and it was capable of degrading pentylamine and aniline. Liu *et al.* (2002) isolated a bacterial strain, AN3, which was able to use aniline as sole carbon and nitrogen source from activated sludge and identified it as *Delftia* sp. AN3. This strain was capable of growing in concentrations of aniline up to 5000 mg l^{-1}. O'Neill *et al.,* (2000) isolated a consortium of bacteria capable of degrading aniline found in wastewaters produced by oil fields, in marine mud and in acid peat bog water and soils.

In the present study, aniline degrading bacteria were isolated from the soil around Shiraz refinery, Iran and identified by physiological and biochemical tests and 16S rDNA gene sequencing. HPLC analysis and MIC test were used to determine aniline biodegradation rate and the most resistant isolated strain, respectively. Furthermore, growth rate and optimum physicochemical parameters for efficient degradation of aniline by superior strain were surveyed.

2. Materials and methods

2.1. Soil sampling

Sampling of soil around Shiraz Refinery, Iran (located at 30° 29` east latitude and 52° 22` north longitude) was done in autumn year 2012, from three stations and each station 3 times. Soil sampling depth was 3 to 5 cm and done by sterile containers and were transported to the laboratory within 3 hours in ice containers.

2.2. Enrichment and isolation of bacteria

Enrichment was performed using a basal salt medium (BSM) containing (per liter) $MgSO_4.7\ H_2O$ (CAS 10034-99-8) (0.5 g), $(NH_4)_2SO_4$ (CAS 7783-20-2) (0.5 g), K_2HPO_4 (CAS 7758-11-4) (0.5 g), $FeCl_3.H_2O$ (CAS 24290-40-2) (10 mg), $CaCl_2.H_2O$ (CAS 13477-29-7) (10 mg), $MnCl_2$ (CAS 7773-01-5) (0.1 mg), $ZnSO_4$ (CAS 7733-02-0) (0.01 mg) and sodium citrate (CAS 68-04-2) (0.01 mg). 95 ml of BSM containing 0.1 g l^{-1} of aniline (CAS 62-53-3) was mixed with 10 grams of soil sample. The pH of the medium was adjusted to 7.0. Cultures were incubated aerobically on a reciprocal shaker (100 rpm) at room temperature (23-25 °C) in the dark to preclude photolysis reactions. All enrichment cultures were subcultured on the same medium at a one week interval. From a one week-old culture, 10ml was transferred to 90 ml of freshly prepared aniline medium. Repeat the transfer process said above every week. After two months enrichment, the liquid medium was plated on solid culture medium and cultured at 30 °C. Morphologically distinct colonies were selected for plate clearance assay (Kafilzadeh and Farhadi, 2015).

2.3. Identification of aniline degrading bacteria

The isolated strains were identified by colony morphology, Gram staining, microscopic form and some biochemical tests including oxidase, catalase, triple sugar iron (TSI), urease, citrate, lactate dehydrogenase (LD), methyl red (MR), Voges-Proskauer (VP) and sulfur indole motility (SIM) (Holt *et al.* 1994).

2.4. The 16S rDNA gene sequencing

In order to confirm the results obtained from the biochemical and morphological tests molecular identification was done by of 16S rDNA gene sequencing. DNA extraction was done with the CinnaGen company kit program (DNP kit, CinnaGen, Iran). For PCR amplification, two universal primers for the Domain Bacteria, 27F 5′-

AGAGTTTGATCMTGGCTCAG-3' and 338R 5'-GCTGCCTCCCGTAGGAGT-3' were used as sense and antisense primers, respectively (Suzuki and Giovannoni, 1996). The reaction mixture was prepared to a total volume of 25 µl containing: 1µl dNTP, 10 µl MgCl2, 10x PCR buffer, 5 µl of each primers, 2 µl template DNA, 2 µl Taq polymerase. The reaction mixture was incubated in a thermal cycler with an initial denaturation step at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 56 °C for 45 seconds and extension at 65 °C for 40 seconds and a final extension step at 72 °C for 10 minutes. PCR product was analyzed in 1% agarose gel (CAS 9012-36-6) stained with ethidium bromide. Gels were photographed by Gel Doc system. DNA sequences of the cloned 16S rDNA fragments compared using BLAST at http://www.ncbi.nlm.nih.gov/BLAST/ (National Center of Biotechnology Infor-mation, NCBI).

2.5. Biodegradation experiments

Aniline degradation experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of BS medium. Aniline was added to BS liquid media to final concentration of 100 mg l^{-1}. Bacteria strains (10^6 cells ml^{-1}) was incubated overnight (~14 h) in 5 ml nutrient broth at 30 °C. After incubation, cultures were centrifuged at 6000 rpm for 15 min and cells collected. These were washed three times with BS liquid medium and resuspended in a small volume (5 ml) of the same medium. Inocula for all experiments were prepared by diluting the recovered cultures with desired media to give a spectrophotometric reading of 0.05 at 600 nm. Cell suspension (500 µl) was inoculated into a 100 ml BS. The cultures were incubated on orbital shaker at 150 rpm at 30 °C for 7 days. At 24 h interval, 20 ml of each culture were sampled for chromatographic analyses. Controls involving the use of uninoculated flasks were included and each experiment was performed in triplicate (Weaver *et al.* 1994; Kafilzadeh and Farhadi, 2015).

2.6. Extraction and determination of aniline

Two ml of methanol (CAS 67-56-1) were added to 5 ml BS medium in screw cap glass tubes that were than shaken several times. One ml of the upper phases (methanol) were transferred to clean tubes and evaporated on a rotary evaporator. The remaining pellets were dissolved in 2 ml of HPLC mobile phase and stored at 4 °C for HPLC analysis. Ten µl of each sample were injected into a Shimadzu HPLC system equipped with UV detector at a fixed wavelength 254 nm. The analysis was carried out by using a C18 column. Methanol: water (80:20 v/v) was used as a mobile phase at a flow rate of 0.5 ml min^{-1} (Weaver *et al.* 1994; Kafilzadeh and Farhadi, 2015).

2.7. The minimum inhibitory concentration test (MIC)

MIC test was used to determine the most resistant isolated bacteria to aniline which is the minimum concentration of aniline that inhibit the growth of bacteria. First, the bacterial suspensions were prepared in LB Broth medium. Then for each bacterium, 10 tubes containing 5 ml basal medium (MS) was considered, then autoclaved and each of the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 g l^{-1} of aniline were added to each tubes. At the end 0.1 ml of the prepared suspension was added to the tubes and incubated at 30 °C for 48 hours. The turbidity was then measured in each of the tubes. The first tube with no turbidity showed the MIC value. According to the results, the best aniline degrading bacterium and the most resistant bacterium to aniline (strain KH-A5) was selected for further analysis.

2.8. Effect of initial aniline concentration on the biodegradation and growth of strain KH-A5

The culture of strain KH-A5 was prepared and adjusted to an optical density at 600 nm (OD_{600}) of 1.0, then the final density of 10^6 cells ml^{-1} inoculums were inoculated into the flasks containing BS media with aniline as sole carbon source. The range of aniline concentrations was increased from 10 to 800 mg l^{-1}. The flasks were incubated at 30 °C with 180 rpm for 7 days. Samples were collected periodically to measure the biomass and the aniline degradation. The biomass contents were monitored spectrophotometrically by measuring absorbance at 600 nm.

2.9. Effect of initial pH on the biodegradation of aniline by the strain KH-A5

The effect of initial pH on the biodegradation of aniline by the strain KH-A5 was also studied in 250 ml conical flasks. Ten ml of strain KH-A5 culture was inoculated into flasks containing 90 mL BS medium with the presence of 100 mg l^{-1} aniline to give the final KH-A5 density of 10^6 cells ml^{-1}. The pH values of medium were then adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0, respectively, using 2 mol l^{-1} H_2SO_4 (CAS 7664-93-9) or 2 mol l^{-1} NaOH (CAS 1310-73-2). These flasks were then shaken on a rotary shaker at 180 rpm and 30 °C. Samples were also collected from each flask at 24 h intervals and centrifuged at 6000 rpm for 15 min before being subjected to determine the aniline concentration.

2.10. Effect of temperature on the biodegradation of aniline by the strain KH-A5

Strain KH-A5 culture of 10 ml was inoculated into 250 mL conical flasks containing 90 ml BS medium with the presence of 100 mg l^{-1} aniline to give the final KH-A5 density of 10^6 cells ml^{-1}. The pH values of medium were adjusted to 7.0 using 2 mol l^{-1} H_2SO_4 or 2 mol l^{-1} NaOH. Then these flasks were shaken on rotary shakers at 180 rpm and five different temperatures, i.e., 15, 20, 25, 28 and 35 °C, respectively. Samples collection and treatment procedures were the same as before.

2.11. Effect of second carbon or nitrogen sources on the biodegradation of aniline by the strain KH-A5

To study the effect of second carbon or nitrogen sources on the degradation of aniline, different dosages (0.5, 1.5, and 3 g l^{-1}) of glucose (CAS 50-99-7) as a second carbon source as well as different dosages (0.5, 1.5, and 3 g l^{-1}) of potassium nitrate (CAS 7757-79-1) as a nitrogen source were added into the flasks containing 90 ml BS medium, 10 ml strain KH-A5 culture and 100 mg l^{-1} of aniline. Only aniline added as sole carbon and nitrogen source was set as control. The pH values of medium were adjusted to pH 7.0 using 2 mol l^{-1} H_2SO_4 or 2 mol l^{-1} NaOH and incubated on a rotary shaker at 180 rpm and 30 °C.

3. Results

3.1. Identification of aniline degrading bacteria

The results obtained from morphological and biochemical tests are shown in table 1. Five bacteria were identified including *Enterobacter ludwigii, Raoultella planticola, Alcaligenes faecalis, Serratia marcescens* and *Microbacterium barkeri*.

Table 1. The results of morphological and biochemical tests

Bacteria	Gram	Shape	Motility	H_2S	Citrate	OD	LD	Urease	Indole	Catalase	Oxidase	VP
E. ludwigii	-	Rod	+	-	+	+	+	-	-	+	-	+
R. planticola	-	Rod	-	-	+	-	+	+	-	+	-	+
S. marcescens	-	Rod	+	+	+	+	+	-	-	+	-	+
M. barkeri	+	Rod	+	-	+	-	-	-	-	+	-	-
A. faecalis	-	Rod	+	+	+	-	-	+	-	+	+	+

The sequences of 16S rDNA of isolated bacteria were compared with sequence of registered bacteria from the GenBank. The homology between sequences obtained from 16S rDNA and gene bank showed *E. ludwigii* with 98% similarity, *S. marcescens* with 99% similarity, *A. faecalis* with 99% similarity, *R. planticola* with 98% similarity and *M. barkeri* with 97% similarity.

The 16S rDNA gene for these bacteria were registered in the NCBI GenBank, and their accession numbers were as *E. ludwigii* strain KH-A5 Accession: KF366298.1, *S. marcescens* strain KH-A4 Acceession: KF366297.1, *A. faecalis*

strain KH-A3 Accession: KF366296.1, *R. planticola* strain KH-A2 Accession: KF366295.1, *M. barkeri* strain KH-A1 Accession: KF366294.1.

3.2. Biodegradation and determination of aniline

Results from HPLC analysis indicated that aniline was considerably degraded by the bacterial isolates. The highest aniline degradation rate (96%) was observed by *E. ludwigii* KH-A5 followed by *S. marcescens* KH-A4 (78%), *M. barkeri* KH-A1 (75%), *R. planticola* KH-A2 (70%) and *A. faecalis* KH-A3 (64%). The biodegradation capability of the strain KH-A5 for aniline was remarkably higher than other strains. Results are graphically presented in Fig. 1.

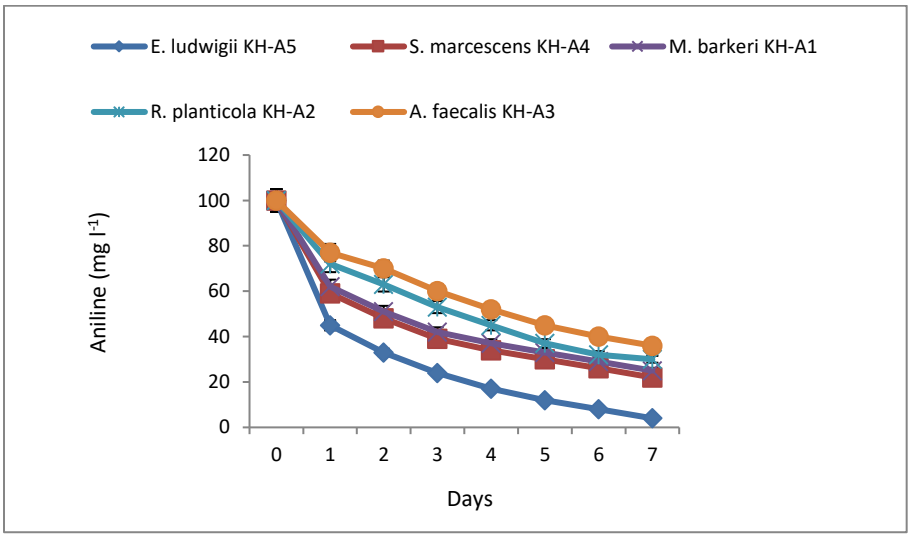

Figure 1. Degradation of aniline by bacteria at 30 °C

3.3. MIC test

The MIC tests results of the various aniline concentrations indicated *E. ludwigii* KH-A5 as the most resistant bacterium. It follows with *S. marcescens* KH-A4, *M. barkeri* KH-A1, *R. planticola* KH-A2 and *A. faecalis* KH-A3, respectively (Table 2).

Table 2. Results of MIC test at 30 °C

	Concentration (g l^{-1})											
Bacteria	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	Test & Bacteria	Test & Aniline
E. ludwigii	-	-	-	+	+	+	+	+	+	+	+	-
S. marcescens	-	-	-	-	-	+	+	+	+	+	+	-
M. barkeri	-	-	-	-	-	+	+	+	+	+	+	-
R. planticola	-	-	-	-	-	-	-	+	+	+	+	-
A. faecalis	-	-	-	-	-	-	-	+	+	+	+	-

3.4. Effect of initial aniline concentration on the biodegradation and growth of strain KH-5

The strain KH-A5 could degrade 59, 77, 96, 71, 53, 40, 31, 19 and 11% of aniline at the initial aniline concentrations of 10, 50, 100, 200, 300, 400, 500, 600 and 700 mg l^{-1}, respectively. The maximum growth and biodegradation of aniline were observed at the initial aniline concentration of 100 mg l^{-1}. There was no growth when the initial aniline concentration was higher than 700 mg l^{-1}. The strain could growth on aniline up to concentration of 700 mg l^{-1} with the degradation rate of 11% (Fig 2).

3.5. Effect of initial pH on the biodegradation of aniline by the strain KH-A5

The strain KH-A5 could degrade more than 50% of aniline in a range of pH levels between 6.0 to 8.0. The optimum pH for biodegradation of aniline by KH-A5 was found to be 7.0. Aniline biodegradation was significantly reduced at a pH 5 and 9 (Fig. 3).

3.6. Effect of temperature on the biodegradation of aniline by the strain KH-A5

As shown in Fig. 4, the optimum temperature for growth of the strain KH-A5 was between 28 and 35 °C. Aniline biodegradation efficiency was the fastest at this temperature range as aniline concentration decreased from 100 mg l^{-1} to 4 mg l^{-1} within 7 days. However, aniline biodegradation efficiency was dramatically decreased when the temperature dropped to 20 and 15 °C.

3.7. Effect of second carbon or nitrogen sources on the biodegradation of aniline by the strain KH-A5

As shown in Fig. 5, the aniline degradation efficiency could reach as high as 96.0% within 7 days when only aniline was added as a sole carbon source.

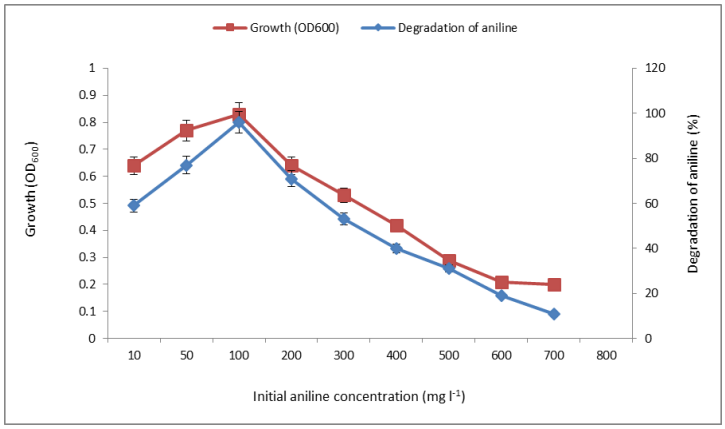

Figure 2. Profile of cell growth and aniline degradation at various initial concentrations of aniline by strain KH-A5 at 30 °C

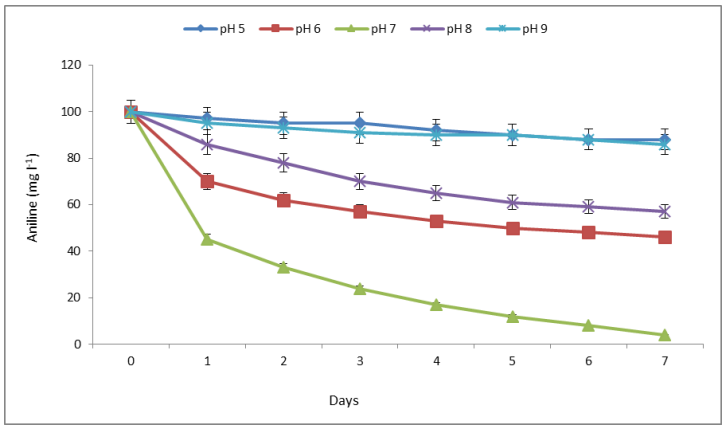

Figure 3. Effect of initial pH on the biodegradation of aniline by strain KH-A5 at 30 °C

After adding 0.5 g l^{-1} glucose as the second carbon source, the aniline degradation efficiency was slightly increased by 3.0% within the same period. However, when more than 0.5 g l^{-1} glucose was added, the aniline

degradation efficiency within the same period even decreased drastically. The same phenomenon could also be observed in the tests of adding potassium nitrate as a second nitrogen source (Fig. 6).

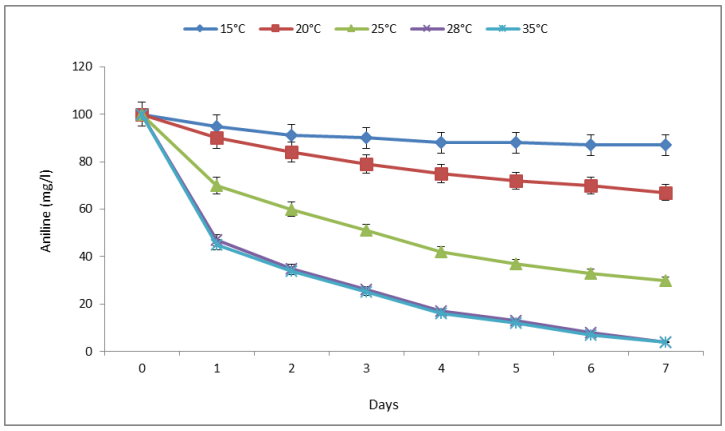

Figure 4. Effect of temperature on the biodegradation of aniline by strain KH-A5 at pH 7.0

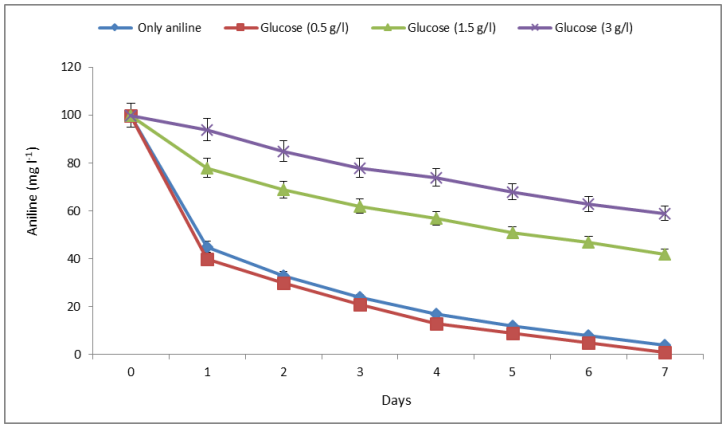

Figure 5. Effect of different doses of glucose as second carbon on the biodegradation of aniline by strain KH-A5 at 30 °C and pH 7.0

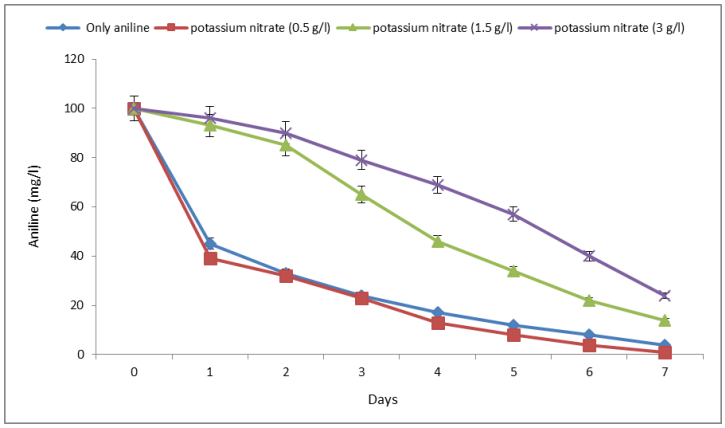

Figure 6: Effect of different doses of potassium nitrate as second nitrogen on the biodegradation of aniline by strain KH-A5 at 30 °C and pH 7.0

4. Discussion

Aromatic compounds are one of the major groups of xenobiotic compounds. Many of these chemicals are persistent, toxic to life forms, and act as stressors. Bacteria are constantly exposed to biotic and abiotic cues, and one such cue is aromatic compounds. One such aromatic compound, aniline, is degraded by different groups of bacteria (Mohammed *et al.,* 2014; Maurice *et al.,* 2013). In the present work, the soil around Shiraz refinery was sampled in autumn year 2012. The colonies of bacteria appeared in the aniline agar medium within 3 to 5 days. It indicates their ability to use aniline as the sole carbon and energy sources. Aniline degrading bacteria were identified via usual biochemical tests and PCR method with 16S rDNA gene sequencing. Obinna *et al.* (2008) identified two strains of *Rhodococcus* isolated from the tropical ecosystem contaminated with aniline, which utilized aniline as carbon resource. Li *et al.* (2010) identified strain HSA6 as *Erwinia amylovora* on the basis of 16S rDNA sequence and morphological and physiological characteristics which was capable of aniline degradation. No *Rhodococcus* and *Erwinia* were found in the current research.

In the present study, different bacteria were used for aniline degradation with varying degrading ability. It was found that the highest degradation rate (96%) was shown by *E. ludwigii* KH-A5 which may be due to favor able environmental conditions as well as high production of enzymes responsible for aniline biodegradation. Ahmed *et al.* (2010) identified strain ST1 as *Staphylococcus aureus* which was capable of degrading 59.65% of aniline after 72 h. Wang *et al.* (2007) isolated strain *Pseudomonas* sp. from activated sludge obtained from a Northeastern China treatment facility for petrochemical wastewater which was capable of degrading 89% of aniline after 24 h at a concentration ranging between 150 and 200 mg l^{-1}. Ahmed *et al.*, (2013) isolated *Corynebacterium* sp. from soil Tahwhay-Monifia (Egypt) which was capable of degrading 30% aniline within 24 h. Xiao *et al.* (2009) isolated *Delftia* sp. XYJ6 that was capable to degrade aniline at rate 100% within 22 h. No *Pseudomonas*, *Staphylococcus* or *Corynebacterium* was found in the current research. During the initial step of cultivation, a sudden drop of aniline level was observed. As demonstrated in figure 1, this rapid decrease of aniline concentration in the medium was due to either its assimilation by bacteria or adsorption onto the cell wall.

In the present research, MIC test was used to determine the most resistant isolated bacteria to aniline. The results indicated that the most resistant bacterium belonged to *E. ludwigii* KH-A5 with MIC of 0.7 g l^{-1}. Moreover, no growth could be observed at the concentrations greater than 0.7 g l^{-1}. It can be concluded that high concentrations of aniline inhibit the growth of bacteria. Kafilzadeh *et al.* (2013) showed that aniline degradation by isolates was considerable and the *Pseudoalteromonas arctica* was the most resistant bacterium with MIC of 0.2 g l^{-1}. Therefore, the tolerance rate of this bacterium to aniline was less than isolated bacteria in the current study. Tolerant bacteria may utilize aniline as source of carbon and energy for their growth and reproduction; furthermore, bacterial metabolism releases enzymes such as glutamine synthetase and aniline dioxygenase, by which aniline is oxidized and consequently, transformed or degraded to less toxic compounds (Arora, 2015).

In the current study, the aniline degradation characteristics and biomass of *E. ludwigii* KH-A5 at various initial concentrations of aniline (10-700 mg l^{-1}) were determined by monitoring aniline concentration and cell growth at OD_{600} periodically. An inhibitory effect showed that the biomass growth and the degradation of aniline were declined with the elevated initial aniline concentration higher than 100 mg l^{-1}. These results was also supported by Li *et al.* (2010), whose isolated *Erwinia* sp. strain HSA6 from activated sludge that was capable of degrading 100% of aniline at 0.5 g l aniline concentration after 24 h culture. The degradation rate of aniline was found to descend as the concentration of aniline increased from 0.5 to 3 g l^{-1}. The growth of strain HSA6 also appeared to decrease with an increase in aniline concentration. Aoki *et al.* (1983) demonstrated that growth of *Rhodococcus erythropolis* AN-13 was strongly inhibited by aniline at concentrations greater than 3.3 g l^{-1}. Decrease in the tolerance rate at higher concentration may be due to toxic effects of aniline with regard to inadequate cell/aniline ratio or biomass through the inhibition of metabolic activity. Similar results were observed in various reported microorganisms (Sarwade and Gawai, 2014).

In the current study, effect of initial pH and temperature on the biodegradation of aniline by the strain KH-A5 was investigated. Aniline concentration was dropped from 100 to 4.0 mg l^{-1} within 7 days especially at pH 7.0, which mean that the biodegradation efficiency could reach as high as 96%. The results are consistent with previous studies which have found that most degrading bacteria, such as *Candida tropicalis* AN1 (Wang *et al.,* 2011) and *Frateuria* (Murakumi *et al.,* 1999), could degrade aniline at a narrow pH range of 6.0 and 8.0. Degradation of aniline also reached the maximal values at a range of temperature levels between 28 to 35 °C. On the contrary, the aniline degradation declined sharply when the temperature dropped to 20 °C and under, indicating that the lower temperature is not beneficial for the biodegradation of aniline by the strain KH-A5. These growth conditions of *E. ludwigii* KH-A5 for aniline degradation were similar those of other bacteria reported previously (Wang *et al.,* 2011; Sarwade and Gawai, 2014).

In the present work, the effect of glucose as a second carbon source or potassium nitrate as a second nitrogen source on the biodegradation of aniline by the strain KH-A5 was also investigated. The results indicated that the addition of 0.5 g l^{-1} glucose or potassium nitrate as a second carbon or nitrogen source could slightly enhance the biodegradation efficiency from 96.0% to 99.0%. However, even more addition of glucose or potassium nitrate could not further enhance the biodegradation process but delayed the biodegradation of aniline by the strain KH-A5. The enhanced degradation of aniline was due to the fact that extra glucose or potassium nitrate could be co-metabolized as a second carbon or nitrogen source to enhance the growth of KH-A5. However, when an excessive second carbon or nitrogen source was present, the degradation of aniline was suppressed since glucose and potassium nitrate could be more easily assimilated than aniline by microorganisms (Jian and Chunjuan, 2007).

5. Conclusions

Five aniline degrading bacterial strains were isolated from the soil around Shiraz Refinery, Iran.

The results show with MIC increase, also increases biodegradation rate. So that *E. ludwigii* KH-A5 has the most degradation rate with the highest MIC. But *R. planticola* KH-A2 and *A. faecalis* KH-A3 had the least degradation rate with the lowest MIC. The optimal pH and temperature range for the biodegradation of aniline by KH-A5 are pH 7.0 and 28-35 °C, respectively. The strain was able to degrade 96% of the initial 100 mg l^{-1} aniline and grow at the aniline concentration of as high as 700 mg l^{-1}. The current study indicates that these strains, particularly *E. ludwigii* KH-A5, would prove to be a promising candidate for bioremediation of aniline contaminated sites.

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