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Petroleum biodegrading and co-resistance to antibiotics by *Serratia marcescens* strain isolated in Coari, Amazonas

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ABSTRACT. *Serratia marcescens* is a Gram-negative bacillus, anaerobic facultative belonging to the family Enterobacteriaceae. *S. marcescens* strains are able to grow in the presence of different xenobiotic compounds, among them, petroleum and heavy metals. Xenobiotic resistant strains develop concomitant resistance to multiple antibiotics, referred to as co-resistance. The AMS212 strain was submitted to the microplate qualitative DCPIP - redox 2,6 dichlorophenol indophenol method. The quantitative test was carried out in Erlenmeyer flasks, followed by the change of color with the absorbance readings, trough the colorimetric method. The antibiotic resistance profile was evaluated by the Kirby-Bauer method. In the qualitative assay, the AMS212 strain altered the color of the DCPIP, which changed from blue to colorless, confirming that petroleum biodegradation occurred. In the quantitative test, the readings were decreasing, confirming that the concentration of DCPIP decreased as a function of the incubation time. The susceptibility test revealed that the AMS212 strain presented multiresistance to four different antibiotics. *S. marcescens* presented high performance in the biodegradation of petroleum, opening possibility to use it in projects involving the remediation of impacted areas. The expression of the antibiotic co-resistance phenotype confirms that the AMS212 strain is able to withstand different environmental aggressions.

Keywords: biodegradation, characterization, DCPIP, antimicrobial resistance.

Biodegradação do petróleo e corresistência a antibióticos por *Serratia marcescens* isolada em Coari, Amazonas

RESUMO. *Serratia marcescens* é um bacilo Gram-negativo, anaeróbio facultativo, pertencente à família Enterobacteriaceae. Linhagens de *S. marcescens* são capazes de crescer na presença de diferentes compostos xenobióticos, dentre eles, petróleo e metais pesados. Linhagens resistentes a xenobióticos desenvolvem concomitante resistência a múltiplos antibióticos, denominada corresistência. A linhagem AMS212 foi submetida ao método colorimétrico com indicador DCPIP - redox 2,6 diclorofenol indofenol, qualitativo, em microplacas. O teste quantitativo foi realizado em frascos Erlenmeyer, acompanhando-se a mudança de coloração, com as leituras das absorbâncias. Avaliou-se o perfil de resistência a antibióticos pelo método de Kirby-Bauer. No ensaio qualitativo, a linhagem AMS212 alterou a cor do DCPIP, que passou de azul para incolor, confirmando que ocorreu biodegradação do petróleo. No teste quantitativo, as leituras foram decrescentes, confirmando que a concentração do DCPIP diminuiu em função do tempo de incubação. O teste de susceptibilidade revelou que a linhagem AMS212 apresenta multirresistência a quatro antibióticos diferentes. *S. marcescens* apresentou alto desempenho na biodegradação do petróleo, abrindo possibilidade de utilizá-la em projetos envolvendo a remediação de áreas impactadas. A expressão do fenótipo de corresistência a antibióticos confirma que a linhagem AMS212 é capaz de resistir a diferentes agressões ambientais.

Palavras-chave: biodegradação, caracterização, DCPIP, resistência a antimicrobianos.

Introduction

Bacteria from *Serratia* genus, Enterobacteriaceae family, are characterized as Gram-negative, facultative anaerobic and chemotrophic bacilli (Hejazi & Falkiner, 1997; Carvalho et al., 2010). They are cosmopolitan bacteria (Ashelford, Fry, Bailey, & Day, 2002), and may be isolated from the aquatic environments, petroleum, animals, including humans and plants (Grimont & Grimont, 1992; Ashelford et al., 2002).

Serratia species have been described in the literature as being able to grow in environments containing petroleum hydrocarbons (Rojas et al.,

2002; Wongsa et al., 2004; Ortega-González et al, 2013), and in environments saturated with heavy metals (Alzubaidy, 2012; Silva et al., 2012; Ibrahim, Syed, Shukor, & Ahmad, 2014). The *S. marcescens* specie has been reported as the most important *Serratia* species due to its ability to produce compounds, like prodigiosin, a natural red pigment, Serrawettina, a biotensoative that gives it adherence in the process of colonization of surfaces, and also enzymes, like as proteases, nucleases, lipases, chitinases, benzonases and cloroperoxidases (Montaner et al., 2000; Pérez-Tomás, Montaner, Llagostera, & Soto-cerrato, 2003; Morohoshi et al., 2007; Kalivoda et al., 2010). Due to the metabolic capacity of *S. marcescens* strains to degrade petroleum, these strains have been used alone or in microbial consortia, aiming at the recovery of environments impacted by petroleum spills and derivatives (Wongsa et al., 2004; Ortega-González et al., 2013; Silva et al., 2015).

Serratia marcescens is able to survive in inhospitable environments, presenting resistance to antiseptics, disinfectants and antibiotics (Aucken & Pitt, 1998; Doi et al., 2004; Iguchi et al., 2014). The ability to metabolize different xenobiotics, produce bioactive compounds as well as colonize different environments are among the characteristics that gives to this species multiple abilities and a great biotechnological and commercial potential. In this context, the present research aimed to study *S. marcescens* AMS212 bacterial strain isolated from the aquatic environment around the Urucu Petroleum Base, Coari - Amazonas and to test its potential to degrade petroleum, and to evaluate its antimicrobial resistance profile.

Material and methods

Area of study, enrichment and isolation

The study area comprises the natural stream (S 04°51'40.4" / W 065°17'52.7"), located near the Urucu, Coari, Amazonas-Brazil Petroleum Base. For isolating the strain, 10 mL of the water sample were incubated in Erlenmeyer flasks of 250 mL with 90 mL of Bushnell Haas (BH) broth (Difco™) commercial medium; 1% of raw petroleum as the source of carbon. The samples were in orbital shaker (Thermo Scientific™ MaxQ™ 4000), 180 rpm min.$^{-1}$, 30°C, during 21 days. Isolation of the microorganism consisted in the use of commercial BH agar containing petroleum as the sole source of carbon. The pure culture was transferred to commercial tryptic soy agar - TSA (Difco™). The petroleum used in the experiments came from the Petroleum Province of Coari and was previously sterilized by the filtration method in millipore® 0.22μm. The AMS212 strain is deposited in the bacterial genetics laboratory of the *Universidade Federal do Amazonas*-UFAM.

Preparation of bacterial inoculum

The inoculum was previously cultured in Nutrient broth (HIMEDIA) at 30°C for 12 hours at 180 rpm in an orbital shaker (Thermo Scientific ™ MaxQ ™ 4000). Cells were centrifuged for 10 min., washed with saline solution (0.9%) and standardized in a UV-VIS (Thermo spectronic Biomate 3) spectrophotometer at 600_{nm} and D.O = 1 (10^9 cells mL^{-1}).

Biodegradability test

Qualitative analysis

The qualitative analysis of petroleum biodegradability was carried out in sterile multiwell (24 wells) microplates, incubated at 30°C. The DCPIP redox indicator [0.01 g L^{-1}] was dissolved in sterile BH broth (Himedia). The evaluations were performed every 12 hours until the color change of the medium occurred. The test was performed as described by Hanson, Desai, and Desai, (1993) and Bidoia, Montagnolli, and Lopes (2010), with adaptations. The assay was assembled in triplicate, according to Table 1.

Quantitative analysis

The quantitative analysis was conducted in 250 mL Erlenmeyer flasks, in triplicate, incubated at 30°C at 180 rpm in an orbital shaker (Thermo Scientific ™ MaxQ ™ 4000). Aliquots of 1 mL of the culture were collected to read the absorbance at 600_{nm} in a UV-VIS spectrophotometer (Thermo spectronic Biomate 3) at 24, 48, 72 hour intervals. For this test, the DCPIP solution was prepared in the concentration of 1 g L^{-1} in sterile BH medium (Hanson et al., 1993; Bidoia et al., 2010, with adaptations). Table 1 shows the composition of this assay.

The quantitative evaluation was performed by reading the absorbance, converted to mg of DCPIP, using the standard curve (R^2 = 0.9998) (Figure 1), following Equation 1: [DCPIP] = ($Abs._{600}$ + 0.0037) / 0.154. Where: [DCPIP] = concentration of redox indicator DCPIP in mg L^{-1}; and $Abs._{600}$ = absorbance of the sample at wavelength 600_{nm}. The standard curve was drawn from five dilutions of the DCPIP indicator in BH broth. Thus, absorbance readings were performed at 600_{nm} UV-VIS spectrophotometer (Thermo spectronic Biomate 3) as shown in Table 3.

Table 1. Composition of qualitative test.

Test	Composition	DCPIP [0.01 g L^{-1}]	Petroleum	AMS212	BH
Control 1	DCPIP+BH	1.5 mL	-	-	2.5 µL
Control 2	DCPIP+BH+Petroleum	1.5 mL	10 µL	-	2.5 µL
Inoculum	DCPIP+Petroleum+AMS212	1.5 mL	10 µL	25 µL	-

Table 2. Composition of the quantitative assay.

Test	Composition	BH	DCPIP	Petroleum	AMS212	H_2O
Control 0	BH	90.000 mL	-	-	-	10.000 mL
Control 1	BH + DCPIP	90.000 mL	4.761 mL	-	-	5.239 mL
Control 2	BH+DCPIP+Petroleum	90.000 mL	4.761 mL	0.595 mL	-	4.644 mL
Control 3	BH+DCPIP+AMS212	90.000 mL	4.761 mL	-	2.380 mL	2.859 mL
Inoculum	BH+DCPIP+Petroleum+AMS212	90.000 mL	4.761 mL	0.595 mL	2.380 mL	2.264 mL

Table 3. Absorbance readings at 600nm and dilutions of DCPIP redox indicator.

Erlenmeyer Flasks	Volumes (mL)			[DCPIP]	$Abs._{600}$ nm
	BH	DCPIP [1 g L^{-1}]	H_2O	(mg L^{-1})	u.a
0	90.000	0.000	10.000	0.000	0.0000
1	90.000	0.595	9.405	5.950	0.0880
2	90.000	1.190	8.810	11.900	0.1730
3	90.000	2.380	7.620	23.800	0.3620
4	90.000	3.571	6.429	35.710	0.5490
5	90.000	4.761	5.239	47.610	0.7270

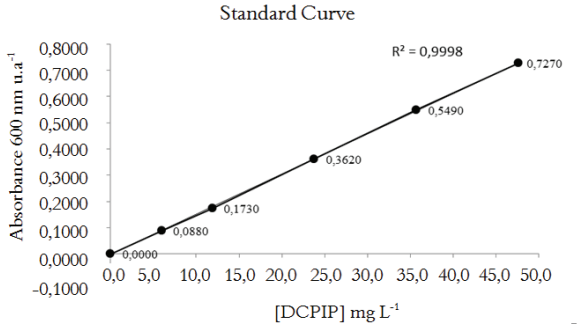

Figure 1. Standard curve - [DCPIP].

Antimicrobial sensitivity test

The sensitivity profile of the AMS212 strain was evaluated by the Kirby and Bauer method (Bauer, Perry, & Kirby, 1960) using pre-loaded antibiotic disks (Laborclin®) according to the manufacturer's instructions. The susceptibility of the AMS212 strain was evaluated against the antibiotics: ampicillin (AMP), amikacin (AMI), amoxicillin plus clavulanate (AMC), ceftazidime (CAZ), cefepime (CPM), cefoxitin (CFO), cefuroxima (CRX), ciprofloxacin (CIP), gentamicin (GEN), meropenem (MER), cephalothin (CFL) and sulfazotrim (SUT). The antimicrobial susceptibility test was performed in duplicate. Escherichia coli ATCC strain was used as negative control.

Phenotypical characterization

The bacterial isolate AMS212 was characterized morphologically and submitted to the biochemical screening system of enterobacteria (Newprov®) which allowed to identify the microorganism at the specie level, based on Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley, & Williams, 1994).

Amplification and sequencing of rRNA 16S g ene

Genomic DNA from the AMS212 bacterium was extracted with PureLink™ Genomic DNA Mini Kit, (Invitrogen™) according to the manufacturer's instructions. The amplification reaction was performed by using primers 530F (5'TGACTGACTGAGTGCCA GCMGCCGCGG3') and 1495R (5'TGA CTG ACTGAGAGCTCTACCTTGTTA3'). The total reaction volume was 25 μL containing (2.5 μL $MgCl_2$ [25 mM], 2.5 μL dNTPs [2.5 mM]; 2.0 μL of each primer [5 pMol μL^{-1}]; 0.3 μL of Taq DNA Polymerase [5 U μL^{-1}]; 2.5 μL of 10X buffer). In vitro amplification of the sequences belonging to the rRNA 16S gene was conducted on the thermal cycler (Thermal Cycler, Veriti® 96-Well - Applied Biosystems). The amplification was conducted in Thermal Cycler, Veriti® 96-Well - Applied Biosystems, covering 35 cycles. The thermal profile of the PCR reaction consisted of the initial denaturation cycle at 95ºC for 1 minute; 95ºC for 40 seconds, primer annealing at 58ºC for 40 seconds and polymerization at 72ºC for 40 seconds (35 cycles); and a final extension step at 72ºC for 7 minutes. For the amplicon sequencing, BigDye® Terminator v3.1 sequencing kit was used in a capillary sequencer (model 3500 ABI PRISM® Genetic Analyzer, Applied Biosystems®).

Phylogenetic analysis

The sequences generated were treated in the phred/phrap (Ewing & Green, 1998) for removal of low quality bases, then only sequences with quality > 20. The analyzes were carried out at the bioinformatics site of the Universidade de Brasília available at http://www.biomol.unb.br/phph/, and then analyzed in the BLASTn program of the National Center for Biotechnology Information – NCBI (Altschul, Gish, Miller, Myers, & Lipman, 1990). The phylogenetic analysis was made based on the evolutionary distances calculated by the Neighbor-Joining and Jukes-Cantor algorithms, using the MEGA 7.0 program (Saitou & Nei, 1987; Jukes & Cantor, 1969). After the construction of the phylogenetic tree, it is possible to determine the taxonomic position of the isolate in relation to the species whose corresponding sequences were obtained in NCBI (Felsenstein, 1985; Kumar, Stecher, & Tamura, 2016).

Results and discussion

The biodegradability test using the DCPIP redox indicator showed the potential of the AMS212 strain in using petroleum as a carbon source. The biodegradation was evinced through the chemical reactions undergone by the DCPIP during the microbial oxidation of the hydrocarbons. The qualitative analysis showed visually that the bacterial strain AMS212 was able to alter the coloration of the (oxidized) blue to colorless (reduced) medium after 16 hours of incubation. The quantitative analysis performed by absorbance reading showed a decline in the DCPIP concentration. The AMS212 inoculum demonstrated a reduction in DCPIP concentration over controls over the 24-hour period (Figure 2). The lack of carbon source in the control (3) showed a low reduction in DCPIP concentration due to low microbial activity in this assay (Table 4). It has been observed that after 48 hours, there was an increase in the concentration of DCPIP. According to Bidoia et al. (2010), DCPIP is a reversible indicator, returning to its original coloration after reduction occurs.

Mariano, Bonotto, De Angelis, Pirôllo, and Contiero, (2008) evaluated the rate of biodegradation of diesel and biodiesel by the colorimetric method with the redox indicator DCPIP, establishing the final time of the experiment by altering the color of the medium, from blue (oxidized) to colorless (reduced). Varjani and Upasani (2013), when assessing the hydrocarbon degradation by a bacterial consortium, recorded the lowest discoloration time of the medium in 120 hours, out of a total of 144 hours. The selection of degrading microorganisms using DCPIP has been a trend and its efficacy has been proved by different authors (Hanson et al., 1993, Mariano et al., 2008, Bidoia et al., 2010, Luz et al., 2011). The species *S. marcescens* has been described as degrading the most diverse types of petroleum hydrocarbons and derivatives (De La Fuente, Perestelo, Rodriguez-Perez, & Falcon, 1991; Ijah, 1998; Wongsa et al., 2004; Jaysree, Rajam, & Rajendran, 2015). Wongsa et al., 2004, emphasizes that the *S. marcescens* species is capable of degrading a broad spectrum of hydrocarbons, mainly aromatic compounds, however, the authors also found the degradation of long chain alkanes. De La Fuente et al. (1991) reports the ability of *S. marcescens* to oxidize aromatic aldehyde compounds.

The antimicrobial susceptibility profile showed that the AMS212 strain was sensitive to most of the antibiotics tested, including amikacin (AMI), ceftazidime (CAZ), cefepime (CPM), cefoxitin (CFO), ciprofloxacin (CIP), gentamicin (GEN), meropenem (MER) and sulfazotrim (SUT), and was resistant to ampicillin (AMP), cefuroxime (CRX), cephalothin (CFL), with particular resistance to amoxicillin plus clavulanate (AMC) confirming the Beta Lactamase of Extended Spectrum (ESBL) (Figure 3). The problem of multiresistance to antibiotics worldwide in bacterial populations of medical importance has led the international scientific community to consider some factors predisposing to the increase of bacterial strains resistant to these antimicrobials (Nikaido, 2009; Singer, Shaw, Rhodes, & Hart, 2016).

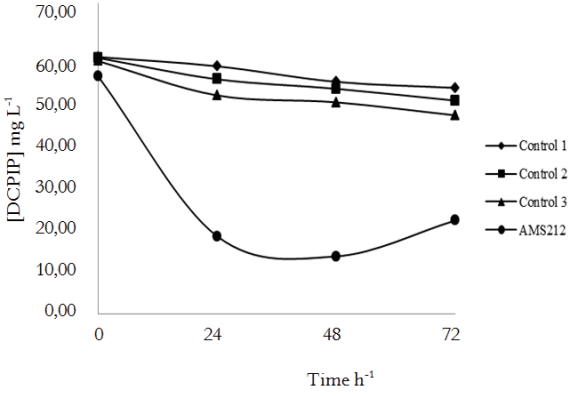

Figure 2. Concentration of DCPIP as a function of time.

Table 4. DCPIP Concentrations.

Time h^{-1}	Control 1	Control 2	Control 3	AMS212
0	57.97	57.77	57.06	53.60
24	55.89	52.97	49.33	17.60
48	52.38	50.82	47.77	13.08
72	51.02	48.16	44.85	21.13

Among the factors that may favor a greater dispersion of antimicrobial resistance genes, environmental contamination by toxic compounds, such as petroleum and other xenobiotics, tends to select bacterial strains capable of surviving high concentrations of these cytotoxic compounds (Kümmerer, 2004; Davies & Davies, 2010; Manaia, Macedo, Fatta-Kassinos, & Nunes, 2016). Moreover, these bacterial strains capable of metabolizing xenobiotics develop co-resistance to one or more antibiotics (Owolabi, & Hekeu, 2014; Thompson, Maani, Lindell, King, & McArthur, 2007), becoming reservoirs of antibiotic resistance genes (Manaia et al., 2016). There are reports in the scientific literature which correlate the ability of strains of *S. marcescens,* recognized as opportunistic pathogen, to resist the presence of heavy metals and biocides, with concomitant multiresistance to antibiotics (Jafarzade, Mohamad, Usup, & Ahmad, 2012; Nageswaran, Ramteke, Verma, & Pandey, 2012). However, the present study correlates the metabolic capacity of the AMS212 *S. marcescens* strain to degrade petroleum, expressing multiresistance to four different antibiotics, including the ESBL.

The morphological characterization of the strain was made with Gram's staining, allowing the AMS212 strain to be identified as a Gram-negative bacillus. In the biochemical tests, the strain AMS212 was negative in the tests of oxidase, L-Triptofano desaminase, hydrogen sulfide, urease, and indole; ferments glucose, but not producing gas; not ferments lactose; was positive for motility, lysine decarboxylase, and Simmons' citrate. The tests allowed identifying the AMS212 strain as belonging to the *S. marcescens* species. Although environmental isolates of the species *S. marcenscens* have been described in the literature as producers of red pigment prodigiosin (Grimont & Grimont, 1984, Hejazi & Falkiner, 1997), AMS212 strain identified in this study did not express prodigiosin production. According to Grimont and Grimont (1984) the ability to produce prodigiosin is characteristic of the *S. marcescens* species, however, the function of this pigment is still unknown, because clinical isolates are rarely pigmented. It can be deduced that the fact that the strain isolated in this study did not produce prodigiosin is related to the cultivation conditions, such as availability of carbon and nitrogen sources, temperature, pH, oxygenation, and luminosity, as suggested by the authors Rjazantseva, Andreeva, and Ogorodnikova, (1994) and Hejazi and Falkiner, (1997). Among other factors that may have contributed to the non-expression of prodigiosin by the *S. marcescens* AMS212 isolate, the strain was submitted to the mineral medium enriched with petroleum as the only source of carbon and energy, which may have caused stress to prevent it from expressing the red pigment. Kim, Lee, and Yim (2009) report that the synthesis of prodigiosin in *S. marcescens* is related to the availability of inorganic salts such as sodium chloride, glycerol as carbon source and ammonium salts as a source of nitrogen.

The amplification and sequencing of the rRNA 16S gene confirmed the identification of the AMS212 strain, after submission of the nucleotide sequences to the BLASTn (Basic Local Alignment Search Tool) program, as belonging to *S. marcescens* specie with 99% identity and Query cover 100% compared to *S. marcescens* strain NBRC 102204 16S ribosomal RNA gene available in the GenBank. The nucleotide sequence was deposited in GenBank database as *S. marcescens* AMS212 under accession number KX686744. Phylogenetic analysis to confirm the taxonomic position of the strain was performed in the MEGA 7.0 program (Felsenstein, 1985; Kumar, Stecher, & Tamura, 2016), aligning the rRNA 16S gene sequence of the isolated *S. marcescens* AMS212 strain (KX686744), along with nine other of rRNA 16S gene of different species of *Serratia* genus obtained in the Genbank. Phylogenetic analysis demonstrated on this study is the sequence *S. marcescens* AMS212, grouped with the sequence belonging to the *S. marcescens* species (288779640_AJ233427.1) available in the GenBank - NCBI, presenting 100% *bootstrap*, when compared with the sequences of other species belonging to Serratia genus, as shown in Figure 2 (Saitou & Nei, 1987).

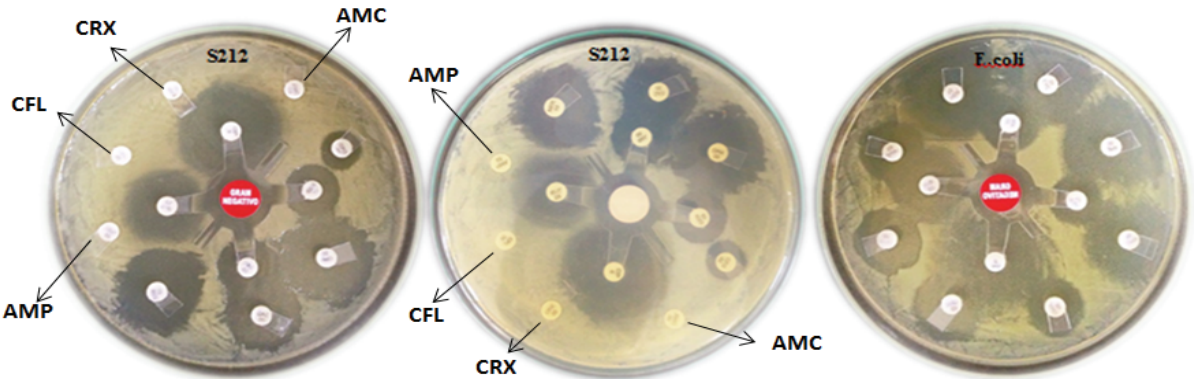

Figure 3. Antimicrobial activity profile of isolated *S. marcescens* AMS212. *E.coli* was used as control.

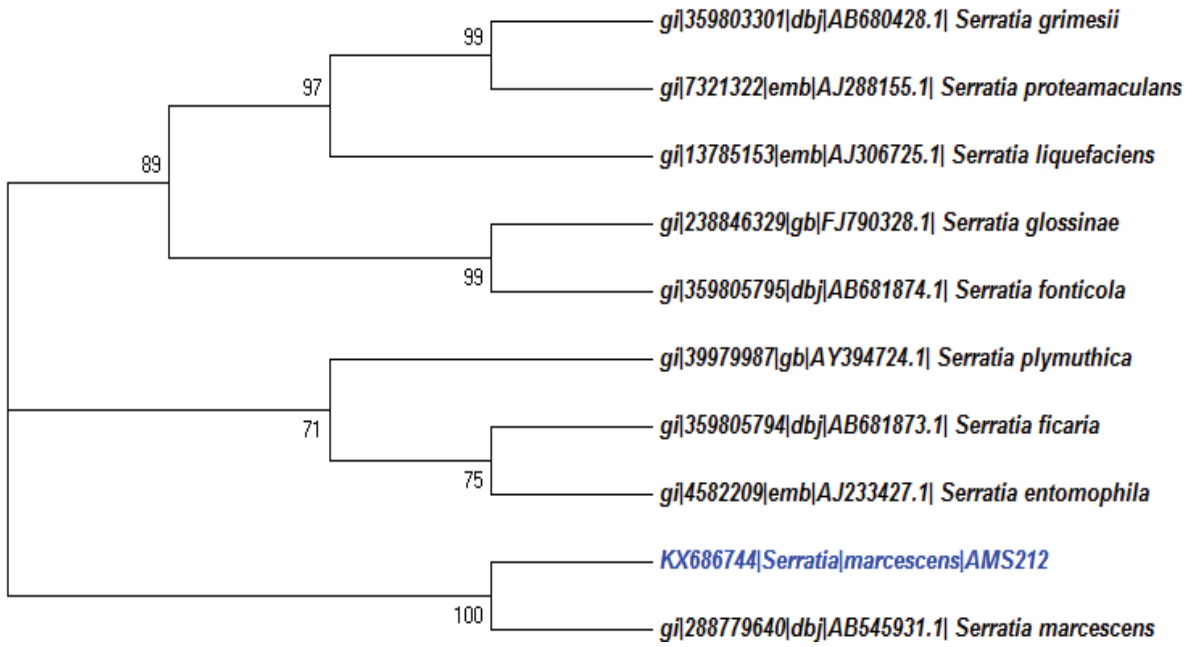

Figure 4. Phylogenetic tree showing the taxonomic location of AMS212 strain. The phylogenetic dendrogram was built by using Neighbor-Joining method, based on sequences from gene region rRNA 16S. Values of bootstrap determined for 1000 repetitions are shown at the ramifications nodes.

Conclusion

The *S. marcescens* AMS212 strain, in the quantitative analysis, decreased the concentration of DCPIP, confirming the metabolic capacity of this strain to biodegrade the petroleum. The evaluation of biodegradability using the DCPIP molecule has proved to be an effective method in the search for new bacterial strains with potential to degrade petroleum. In the antimicrobial susceptibility test, *S. marcescens* expressed multiresistance profile to four different antibiotics, confirming co-resistance with petroleum. The AMS212 strain demonstrated biodegradability potential and could be used in future biotechnologies for the remediation of contaminated environments.

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