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MOLECULAR SURVEYING AND RECOGNITION OF BACTERIA DECOMPOSING THE OIL COMPOSITIONS

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ABSTRACT

Polycyclic aromatic compositions are among oil pollutants which Benzene cycles are used in their molecular structure. These compositions are placed in the priority of pollutants by United States environmental protection agency, because of their poisonousness, mutagenesis and carcinogenesis. Among the methods of removing the oil compositions, are biological methods which are often studied more than other methods due to their simplicity and being economic. The aim of this study is molecular survey and recognition of bacteria decomposing the oil compositions. The samples were collected from the soil polluted with oil materials. Fives strains of hot positive bacteria were separated from the soil samples and then it was proceeded to recognize them using recognition kits and molecular method.At first, the separated strains were classified using biochemical, physiochemical and accessible shape detection. Then, the 16S rDNA gene sequence analysis-based molecular recognition was carried out. Separation of Bacteria strains DNAs was replicated using PCR method. The strains were recognized based on 16S rDNA sequence analysis.

Keywords: Decomposing bacteria, biologic decomposition, molecular recognition, hydrocarbon

INTRODUCTION

As the volume and variety of the environmental pollutants increase, environmental protection and protecting the natural sources have been considered more than ever [1]. Polycyclic aromatic hydrocarbons are a group of aromatic compositions having continuous Benzene cycles which are connected linearly, angularly or in clusters. The underground water sources, deposits and soils polluted with oil compositions usually include a mixture of polycyclic aromatic hydrocarbons and other aromatic compositions [2]. These compositions have particular importance in terms of stability in different environments. The main factors for their production are human's industrial activities like incomplete fuel of organic materials during the process of heat decomposition, pouring of oil from tankers, refineries, and excavating oil wells. They are also obtained from natural events like firing in jungles, volcanic activities and some of them are obtained due to processes having biologic origins and sources [3]. These compositions are among potential chemical materials which are dangerous for the environment and they cause hygienic concern. Often polycyclic compositions have undesirable poisonousness features, mutagenesis and carcinogenesis characteristics [4]. Polycyclic aromatic hydrocarbons having light molecular weight are more harmful for fishes and aquatics more than human. United States environmental protection agency has categorized the aromatic hydrocarbons as initial pollutants since 1970 [5]. Hence, recovery, purification and disposal of these materials are of high importance [4].

In recent years, wide researches have been carried out to find the influential methods for removing the oil pollutants. Traditional physical methods including floating systems, pan holders and absorbents are not able to collect more than 10 to 15 percent of the leaked crude oil. Also application of chemical surfactants as cleansing factors is not very considered because of poisonous effects on marine alive creatures [6]. Necessity to environment-friendly technologies has considered usage of micro-organisms to remove oil compositions. In addition, biologic methods are more economical than physical and chemical methods [7]. Micro-organisms can grow under variable conditions of pH, temperature, available nutrients and high concentration of pollutants. Cheapness, abundance and availableness, production of slight value of biologic and chemical sludge, possibility to revive the bio-absorbent and lack of producing secondary pollutions and environmental-friendly are among other advantages of these absorbents. Biologic decomposition is a process which occurs naturally and in appropriate conditions using micro-organisms decomposing oil compositions which are able to consume crude oil hydrocarbons as the source of metabolic carbon [8].Micro-organisms decomposing the hydrocarbons have found compatibility to grow and develop in polluted environments [9].

During the results of the studies in aquatic and earthen environments, micro-organisms including funguses, bacteria and yeasts are among the factors of decomposing the oil hydrocarbons. The most important factor in biologic decomposition is to access the bacterial isolations which are active to decompose the oil compositions [10].Oil crude decomposition includes a series of chained reactions in which the proprietary micro-organisms perform initial attacks to the oil compositions. This process results in producing the moderate-level compositions which would be consumed by another class of micro-organisms. Indigenous micro-organisms (IMO) available in soils and underground waters of oil fields which are used to these difficult conditions during thetime may consume the carbon existing in the pollutants as energy and grow. These bacteria convert the oil compositions to final products, i.e. water and carbon oxide [11].Among the micro-organisms, the bacteria are employed as a capable and efficient biologic absorbent due to having high surface to volume ratio and abundance of attendance in different environments, ability to grow under controlled conditions and accordance to most of the environmental conditions and ability to produce macro-molecules called "Extracellular Polymeric Substances". The major sources of micro-organisms resistant to poisonous compositions are polluted sites. Therefore, extraction of local micro-organisms from the polluted places is essential to purify the oil compositions biologically [1].A wide group of bacteria including Pseudomonas, Acinetobacter, Alcaligenes, Nocardia and Rhodococcus have the ability to decompose the oil compositions biologically [12].

Many studies have proceeded to investigate on the biologic decomposition of a variety of aromatic hydrocarbons by bacteria. Cao et al. [12] used the Pseudomonas bacterium to decompose an abundance of oil compositions. [13]Separated the Alcanivorax bacterium from the waters polluted to the oil in the Bohai Sea, China [14]. Separated a strain of Alcanivorax from the coastal area of the island of Guimaraes in Philippines which was able to decompose the components of heavy crude oil.

[7] Proceeded to molecular recognition of bacteria decomposing the isolated diesel.[10] proceeded to separate the bacteria decomposing the hydrocarbons. [9] proceeded to molecular surveying on the isolated aromatic hydrocarbon decomposing bacteria from polluted soils in Dammam, Saudi Arabia. Mano other studies are carried out in the field of

bacterial decomposing of oil compositions and other pollutants.

Although it has been assigned numerous studies, decomposition of oil compositions by bacteria is among important problems and currently, investigations on the existence of native oil eater bacterial strains in each region and their performance in oil hydrocarbon decomposition are of particular importance. Therefore, the present study has proceeded to molecular investigation and recognition of bacteria decomposing the oil compositions.

MATERIALS AND METHODS

Sampling from polluted soil

The polluted soil is collected with an amount of 5 gfrom different earths polluted by oil compositions in the depth of 0-30 meters below the soil level. The samples were maintained polyethylene containers and transferred to the laboratory under a temperature of -20º C.In order to provide bacterial suspension and enrich the bacteria of the oil samples from non-carbonic environment, 1 g of polluted soil was added to 10 ml of water understerile conditions. Then it was incubated in a temperature of 28º C and shaken with a rate of 150 rpm for 24 h.

Separating the bacteria from the soil

After providing initial bacterial suspension to enrich bacterial culture and bacterial separation, 1 ml of suspension was inseminated in Nutrient agar culture environment. The plates were incubated in a temperature of about 37º C for 24 h, then the constituted colonies were grouped based on morphology, level of growth and color and recognized using standard biochemical methods applied in the study of [15].

Sub culturing

The isolated bacteria were cultured for a short period in order to purify the strains isolated in the nutrient agar slant environment. The bacteria were inseminated using a sterile loop in nutrient agar slant and they were incubated in the temperature of 37º C for 24 h. slant bottles encompassing bacteria were maintained in the refrigerator in the temperature of 4º C before biochemical and recognition tests of separated strains.

BIOCHEMICAL TESTS

Indole test

Insemination was carried out from isolated bacterial colonies into the test tubes containing Tryptone Broth. The test tubes were incubated in the temperature of 37º C for 48 h. 1 ml of Choloroform was added to the tubes. The test tubes were shaken moderately and then Kovac reagent was added to them and shaken slowly for 20 min. Formation of red color in the above layer represents positivity and the yellow color represents negativity of the test.

Catalase test

At first, a drop of sterile physiology serum is placed on the lam and then in the next stage, by adding a drop of oxygenated water to isolated strain colonies, we allow them to be mixed completely. Creation of bubble indicates the existence of catalase enzyme and in the other words, positive catalase.

Oxidase test

A clean paper filter smeared to Tetramethyl-P Phenylenediamine Dihydro chloride substrate is placed on the surface of a plastic sterile plate and then wetted by sterile still water. An amount of the colonies of the isolated strains is added to filter paper and then a change occurs in the color to be dark purple which indicates positive oxidase.

Initial recognition of isolation bacteria

The morphology of cells was surveyed using Gram strain method. According to this method, positive Gram Coccus and negative Gram Coccus bacteria are considered as polycyclic hydrocarbon decomposing bacteria (PHDB). In particular, they include Streptococcus and Corynebacterium. Phenotype categorization of bacteria including cellular morphology, encompasses mobility and color of colonies.

Molecular recognition of bacteria

For molecular recognition of bacteria, the 16S rRNA method includes five sections: DNA extraction, proliferation of 16S rRNA piece by PCR, electrophoresis of PCR product, determination of the sequence of the proliferated piece and finally, complete recognition of micro-organisms. 24-h culture of bacteria was used to extract DNA and the steps of DNA extraction was carried out based on kit DNA extraction procedure. After extracting the DNA, general primers of 27F and 1492R were used in the step of proliferation of the 16S rRNA piece by PCR. Table 1 has illustrated the general primers to proliferate the 16S rRNA part of the bacteria.

The length of the multipliable piece is estimated about 1500 bps. The environment prepared for PCR has a volume about 125 ml and its characteristics is given in table 2.

Table1: General primers for proliferation of the 16S rRNA part of bacteria

Primer name	Sequence of primer	Temperature of link
27F 1492R	5' AGAGTTTGATCMTGGCTCAG 3' 5' GGTTACCTTGTTACGACTT 3'	T_m: 53

Table 2: Components applied in the compositions prepared for PCR

Materials	Sterile still water	PCR buffer (10X)	Magnesium Chloride (25 mM)	dNTP (10mM)	F primer (20 pmole/μl)	R primer (20 pmole/μl)	Taq DNA Polymerase enzyme	DNA pattern
Volume	83/5 μl	12/5 μl	7/5 μl	5 μl	2/5 μl	2/5 μl	1/5 μl	10 μl

After preparing the proliferation solution and negative control which included all components except DNA pattern and placing them in the PCR system, the reactions of proliferating the 16S rRNA piece by PCR was carried out through the below program:

- DNA initial denaturing reaction in the temperature of 94º C for 5 min during a cycle.
- 35 cycles are: DNA denaturing in the temperature of 94º C for 1 min, adhesion of primers to DNA fibers in the temperature of 54º C for 30 seconds, proliferation and increase in the length of DNA fibers in the temperature of 72º C for 1 min and 40 s.
- Finally, an additional cycle was carried out in order to increase the final length of the proliferated fiber in the temperature of 72º C for 15 s.
- Chillness of solution to the temperature of 15º C.

The appeared ribbons were recovered after running the PCR product on 1% Agarose gel and 1% TAE buffer. After observing the bound, the PCR product was cut from the gel and it was sequenced after compressing. In order to match and find the sequence of DNA in pTZR57R/T conveyor, ligation reaction was carried out beside this conveyor. Sample sequencing was carried out after performing confirmation tests like PCR colony, plasmid extraction and enzyme digestion [16].The considered sequences were carried out in NCBI (national center for biotechnological information) and Blastn (basic local alignment search tool for nucteotide). Then, according to elementary recognition of micro-organisms, the type and strain of bacteria was recognized.

Decomposition of Hydrocarbons

Decomposition in liquid environment

Isolations isolated in the liquid culture environment of lysogeny broth including 1% crude oil were impregnated in the temperature of 37º C. Decomposition rate of crude oil hydrocarbons was carried out through weighing process every week in the month. Non-bacterial insemination culture environment was used in the conditions of performing the test s test controls.

Polluted soil decomposition

10 g of dried polluted soil was sterilized in the temperature of 121º C for 1 h.The sterilized soil was mixed with treated oil solution so as to obtain soil with a 1% concentration of crude oil. The soil was inoculated with 1% inoculum, and then cultured at 30 ºC for a month. The oil degradation rate in the soil was determined by the weighting method each week, and germfree soil was considered as the control.

RESULTS AND DISCUSSION

Screening, separation and recognition of bacteria

In this study, the bacteria were separated from the samples of soil polluted with oil compositions of 5 strains. Based on the observations carried out on the grown appearance of micro-organisms, all purified micro-organisms were bacteria. Initial detection tests represented that all isolated bacteria are positive Gram and positive Catalase. 2 strains of bacteria decomposing the hydrocarbons were selected from the oil-polluted lysogeny broth environment based on their growth and colony type. These strains are called S1 and S2 in the study.

Morphology, biochemistry and physiology of the strains were carried out to recognize the bacterial strains along with molecular recognition using 16S rRNA sequence analysis method. Figure 1 has represented the morphology of the isolated strains from polluted soil.

Lysogeny broth culture environment is developed similarly for both strains and it has created greater colonies and this is because of rapid growth and the size of greater colonies.

Physiologic and biochemical tests represented that both strains are positive Gram, positive Indole. The S2 strain has negative oxidase. Both strains are positive catalase. Table 3 has represented the results of physiology and biochemical tests.

Figure 2 has represented the PCR product related to proliferation of the gene encrypting this piece.

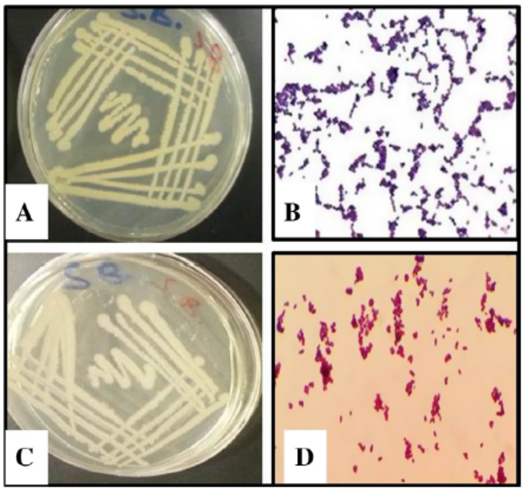

Fig1. Morphology of strains isolated from the sample of polluted soil

Table 3: Morphological features and biochemical and physiologic features of strains isolated from polluted soil

Strain	Morphological features		Physiological and biochemical tests			
	Colony form	Cell shape	Gram stain	Indole	Catalase	Oxidase
S1	Irregular	Rounded	+	+	+	-
S2	Circular	Rounded	+	+	+	++

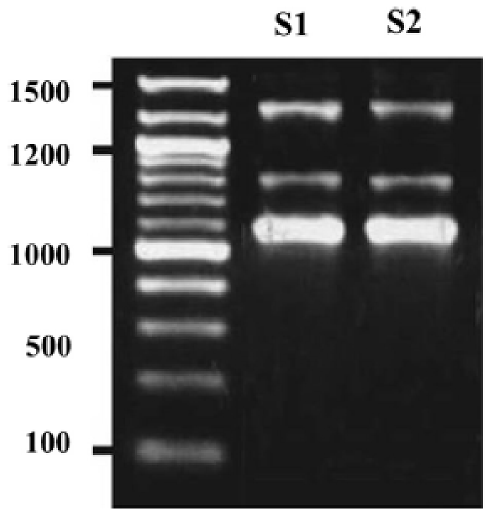

Fig 2: Agarose electrophoresis of 16S rDNA PCR products of the isolated strains Staphylococcus aureus and *Corynebacterium amycolatum*

Table 4: Molecular recognition of the strains isolated through PCR method

No. of strain	Total length (bp)	Gene bank accession no.	Identification result	Similarity (%)
S1	1446	KJ081495.1	*Staphylococcus aureus*	99
S2	1454	KJ081492.1	*Corynebacterium amycolatum*	99

Results of PCR represented that all fragments are significantly comparable to the results of 16S rDNA sequence in order to recognize the bacterial strains.

Based on both methods (biochemical, physiologic and morphology tests) and PCR method, the S1 strain was recognized as Staphylococcus aureus and the S2strain was recognized as *Corynebacterium amycolatum*.

Also, Prathiba et al, (2014)proceeded to molecular recognition of bacteria decomposing the isolated diesel in a study. They separated 9 bacteria from a sample of soil polluted to Diesel in which among these 9 isolations, 3 bacteria had the maximum potential to decompose the diesel. The considered isolations were surveyed through PCR method. Their results recognized the *Pseudomonas aeruginosa* type as decomposing the diesel.

Although the method of their work is similar to the present study, they obtained some differences. It seems the type of oil composition has relationship with decomposing bacterium.[17] reported the separation and detection of bacterium decomposing Phenanterene called Sphingomonas and stated that this bacterium is also able to decompose other cyclic compositions.

[18] investigated on Rhodococcus type cold-friendly bacterium separated from the underground water which is able to grow under the temperature of 4º to 30º Celsius and is able to decompose the hydrocarbons including crude oil, diesel oil and gasoline.

Decomposition of hydrocarbons in liquid environment and soil

A pronounced dissimilarity was recognized in the hydrocarbons degradation rate among the studied strains particularly the S2 C. Figure 3 has represented the isolations decomposing the oil hydrocarbons in liquid environment and polluted soil.

The results represented that in general, the rate of main decomposition of strains increases in the liquid environment over time and the S2 strain was obtained more than 55% in 28 days of the process period and 38% for S1.

Decomposition of hydrocarbons in the polluted soil represented that the rate of decomposition in oil compositions having S2 strain is higher and it reached 70% in a 28-days period. The rate of decomposition by S1 strain was obtained 30 to 40 percent.

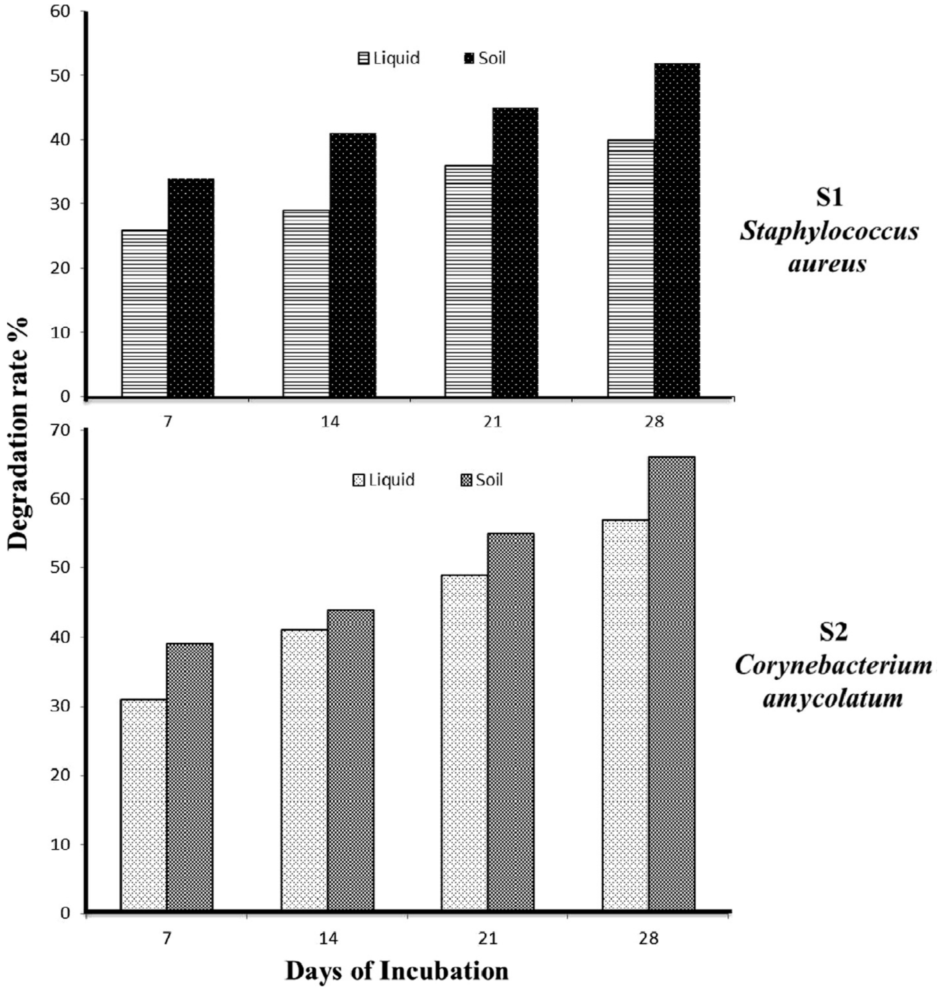

Fig3. The rate ofdecomposing the oil hydrocarbons of S1 and S2 isolated strains in liquid environment (A) and in the environment of polluted soil (B)

CONCLUSION

According to this point thatthe appropriate and effective treatment technologies are essential for oil compositions, today biologic modification is considered as an effective and efficient technology in the process of cleaning the oil hydrocarbons using hydrocarbon decomposing micro-organisms.

Generally, the results represented that 2 strains of bacteria were isolated from polluted soil samples. The separated isolations were based on Staphylococcus aureus and Corynebacteriumamycolatummolecular recognition methods. Also, the experiments of tested biologic decomposition of bacterial strains have represented better ability to decompose the aromatic hydrocarbons. Hence, these bacteria may be used in biologic decomposition processes to clean polluted environments.

REFERENCES

1. S. X.; Xu Y; Li G;Zh. Y; Huang T; and Hu Z. Isolation,

characterization of Rhodococcus sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. Marine Pollution Bull. 62(10):2011. 2122-2128

2. S,J.S.; Y.S.,K.; and Q.X.,Li. Bacterial degradation of aromatic compounds. Int. J. Environ. Res. Public Health. 6: 2009. 278- 309
3. Zh. H.; W. L; R. J. Isolation and characterization of phenanthrene degrading strains Sphingomonas. ZP1 and Tistrella. ZP5. Journal of Hazardous Material. 152 (3):2008. 1293-1300.
4. K., J. A.; R., SH.A. Isolation and characterization of microorganism from oil contaminated sites.Adv. In Applied Sci. Res., 2(3):2011.455-460.
5. S. S; B. L; P. G. Polycyclic hydrocarbons (PAH) slurry phase bioremediation of a manufacturing gas plant (MGP) site aged soil. Water Air and pollution; 134: 2002. 219-236.
6. T. R; J. S; B. IM. Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of Bacillus megaterium, Corynebacteriumkutscheri and Pseudomonas aeruginosa. Bioresour Technol. 2011; 1: 25-27
7. P., G.; D., A.;& B., S. Molecular identification of the isolated diesel degrading bacteria and optimization studies. Journal of Biochemical Technology, 5(3),2014. 727-730.
8. H., M.; Z., M. S.& M., F. H. Isolation and characterization of crude oil degrading bacteria from the Persian Gulf (Khorramshahr provenance). Marine pollution bulletin, 82(1),2014. 39-44.
9. I., M. M.; A., A.; A., D.; Arif, I. A.& E., G. A. Molecular application for identification of polycyclic aromatic hydrocarbons degrading bacteria (PAHD) species isolated from oil polluted soil in Dammam, Saud Arabia. Saudi Journal of Biological Sciences.2015.
10. U., M.; Sh., N., &Jadhav, M. Enrichment, Isolation and Identification of Hydrocarbon Degrading Bacteria. Int. J. Curr. Microbiol. App. Sci, 4(6), 2015.708-713.
11. A. S; R., F. TPH Bioremediation by Microbial Consortium Isolated from Oil Contaminated Soil", 6th International Chemical Engineering Congress & Exhibition.2009.

12. C. B; N. K & C., K. Biodegradation of aromatic compounds: current status and opportunities for bimolecular approaches. Applied Microbiology and Biotechnology 85: 2009.207-228.

13. L. YC; L. LZ; W.Y; T. W; Z. LP; X. L, S. QR, &Sh. Isolation of an alkane-degrading Alcanivorax sp. Strain 2B5 and cloning of the alkB gene. Bioresour Technol. 101: 2010. 310-316.

14. Y. T; M. K; U. S; K. J; M. H; H. M,& S. RB. Biodegradation of heavy oil by Alcanivorax sp. a1 strain isolated from recovered Bunker oil spilt in the “Solar I” accident. MemFac Fish Kagoshima Univ. 2010; 67-73.

15. P., E.J.; B., G.D.; W., J.M.,& Read, D.J. Bacteria associated with Pinussylvestris – Lactariusrufusectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytol. 151, 2001.743–751.

16. S. J & R. DW. Molecular Cloning a Laboratory Manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.2001.

17. S. N.; P. P.; P. K; T. S; O. T & J. K. Isolation and characterization of a phenanthrene degrading sphingomonas sp. Strain P2 and its ability to degrade fluoranthene and pyrene via cometabolism. Research Article Science Asia 27: 2010. 21-28.

18. H. R, J. YH, Y., J. & S. K, Isolation and characterization of Psychrotrophic and halotolerant *Rhodococcus*sp. Yhlt-2. J. Microbiology and Biotechnology, 16 (4): 2006.605-612.