Diesel Degradation and Bioemulsifiers Production Using Bubble-Column with a Microbial Consortium Isolated from Hydrocarbon-Contaminated Soil

Hernández-Martínez, Ricardo; Quiñones-Muñoz, Tannia A.
CONACyT-Instituto Tecnológico Superior de Tierra Blanca. Av. Veracruz S/N Esq. Héroes de Puebla, Colonia Pemex. C.P. 95180. Tierra Blanca, Veracruz, MÉXICO

Vázquez, Adriana
Instituto Tecnológico de Ciudad Madero, Av. 1o. de Mayo esq. Sor Juana Inés de la Cruz s/n Col. Los Mangos C.P.89440, Cd. Madero Tamaulipas, MÉXICO

Lizardi-Jiménez, Manuel A.*[+]
CONACyT-Instituto Tecnológico Superior de Tierra Blanca. Av. Veracruz S/N Esq. Héroes de Puebla, Colonia Pemex. C.P. 95180. Tierra Blanca, Veracruz, MÉXICO

ABSTRACT: *Diesel is composed of various toxic compounds that can have a negative influence on the environment including plants, microorganisms, and even groundwater being used for cultivation and human consumption. Diesel oil biodegradation kinetics was investigated using bubble-column reactor and microbial consortium isolated from a hydrocarbons spill site and were assessed by gas chromatography. The purpose of this study was to demonstrate the importance of intrinsic microorganisms used to degrade diesel. 93.84% of the diesel got consumed in the bubble-column reactor after 15 days of culture. The consortium showed the ability to produce emulsifiers using diesel oil as its only carbon and nitrogen source (hydrocarbonclastic). This study showed that the hydrocarbonclastic consortium isolated from polluted soil has the metabolic tools for diesel degradation (as a single carbon and energy source), and the capacity to produce bioemulsifiers in a bubble column reactor. Microbial consortium and bioemulsifiers produced in this research have the potential to be used in the cleanup processes of polluted soil with hydrocarbons such as diesel.*

KEYWORDS: *Bubble-column reactor; Oil Biodegradation; Bioemulsifiers; Microbial consortium.*

INTRODUCTION

The use, transportation and exploitation (development of hydrocarbon industry, refining, extraction) of hydrocarbons are activities that currently have generated serious problems of environmental pollution causing

** To whom correspondence should be addressed.*
+ E-mail: chamarripas@yahoo.com
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severe ecological impacts in the entire world [1-3]. Particularly, diesel is composed of various toxic compounds that can have a negative influence on the environment including plants, microorganisms and even groundwater being used for cultivation and human consumption [3, 4]. Microorganisms from impacted sites have developed strategies to survive and consume hydrocarbons [3-5]. In order to reduce the effects of oil pollution, various efforts have been made to find a strategy to reduce the impact of pollution in spill sites using different technologies among which are rizhoremediation (plant roots and rizhobacteria) and the use of microorganisms [3, 5, 6]. However, information regarding microorganisms isolated from oil-impacted sites are scarce [1, 2, 5]. Diesel and other hydrophobic compounds have poor solubility in water and resist biodegradation by microorganisms. An approach to solve this problem is the presence of bioemulsifiers, which improve oil degradation in contaminated soils. However, degradation of diesel with microorganisms is the most used strategy due to its ability to use hydrocarbons as carbon and nitrogen sources. In addition, the easy operation of the culture conditions can increase growth or biodegrading rates, this being a solid base for clean-up. Mono and mixed cultures can be used for these purposes [1, 4, 7].

Technologies that use microorganisms have certain advantages. Among them, we can highlight the ability to produce extracellular surface-active compounds (bioemulsifiers) and enhancing the hydrocarbons' biodegradation. Some authors reported that these compounds increase the availability of substrates by increasing the solubility or dispersion [6-8]. Bioemulsifiers are polymers, totally or partially extracellular, amphipathic molecules containing polar and non-polar moieties which allow them to form micelles that accumulate at the interphase between liquids of different polarities such as water and oil thereby reducing surface tension and facilitating hydrocarbon uptake and emulsification [9].

Some microorganisms, though, cannot produce bioemulsifiers but are still able to degrade oil substrates effectively via formation of extracellular or cell membrane-bound bioemulsifiers (such as exopolysaccharides, EPS). Bioemulsifying EPS can enhance microbial hydrocarbon biodegradation in liquid media and soil microcosms [10, 11].

A number of microorganisms have been studied for the production of bioemulsifiers. Extracellular excretions of bioemulsifiers by bacteria have been reported by many scientists [12-14]. Among the bacteria, *Pseudomonas sp.* are best known for degrading hydrocarbon and producing bioemulsifiers mainly rhamnolipid in nature [15].

The yeast *Trichosporon asahii*, isolated from petroleum-contaminated soil in India was found to be the potent producer of bioemulsifier (sophorolipid) in mineral salt media containing diesel oil as the carbon source and found to be an efficient degrader of diesel oil (95%) over a period of 10 days [16]. *Streptomyces sp.* S1 isolated from Goa, India showed maximum bioemulsifier production of 200 EU/mL at time 14 days [17]. Gram-negative bacteria (Acinetobacter, Pseudomonas, Alkanivorax and related genera) are often dominant in microcosms after oil spill simulations [18, 19] and in hydrocarbon contaminated environments after biostimulation [20, 21].

Pseudomonas aeruginosa strain SP4, isolated from petroleum-contaminated soil in Thailand, was used to produce a bioemulsifier (rhamnolipid species) from a nutrient broth with palm oil as the carbon source. When compared to synthetic surfactants, including Pluronic F-68 and sodium dodecyl sulfate, the crude bioemulsifier showed comparable surface activities. The crude bioemulsifier reduced the surface tension of pure water to 29.0 mN/m with a critical micelle concentration of approximately 200 mg/l, and it exhibited good thermal and pH stability. The crude bioemulsifier also formed stable water-in-oil microemulsions with crude oil and various types of vegetable oils, but not with short-chain hydrocarbons [12].

Rahman et al. [15] found methods to increase the biodegradation rates of hydrocarbons from gasoline contaminated soils through ex situ bioremediation. In this study, bacterial growth, hydrocarbon degradation and growth parameters of Phaseolus aureus RoxB were measured. Approximately 67% and 78% of the hydrocarbons were effectively degraded within 60 days in soil samples amended with red soil, gasoline-spilled soil, mixed bacterial consortium, poultry litter, corn pith, and rhamnolipid bioemulsifier at 0.1% and 1%.

Microbial consortia hydrocarbon degraders can be grown in pneumatic bioreactors. Bubble-column have important advantages over stirred bioreactors: reduces cell damage, has higher aeration rates, a larger mass transfer capacity, higher liquid surface velocity and gas flow, simpler construction and lower energy costs. Then,

hydrocarbon-degrading microorganisms with impacted-sites remediation purposes could be cultured in bioreactors [22, 23].

The aim of this work was to evaluate the degradation of diesel and emulsifiers production using a bubble column reactor with a microbial consortium isolated from hydrocarbon-polluted soil.

EXPERIMENTAL SECTION

Hydrocarbonclastic consortium acclimation

A native consortium was obtained from the hydrocarbon spill site, located close to Tancochín River Township Naranjos-Amatlán, Veracruz (21°21'16.0 " N, 97°41' 40.0 " W), this township has an active oil field and are impacted by hydrocarbon pollution. The consortium obtained was acclimatized using Erlenmeyer flasks of 500 mL containing a mineral medium (g/L): KCl 1.13, $NaNO_3$ 6.75, $MgSO_4$*$5H_20$ 0.54, K_2HPO_4 2.15 at a pH value of 6.5 with 13 g/L of fuel diesel as the sole carbon source an aeration of 15 mL of air per second. The flasks were inoculated with 1 g/L of centrifuged inoculum. After 14 days the biomass produced was used for inoculation, the second cycle in acclimation (inoculum size of 10% of total volume) in Erlenmeyer flasks of 1 L containing mineral medium mentioned above and the pH was adjusted at a similar value. The two cycles were conducted at 28°C for 15 days.

Hydrocarbonclastic capacity

A 1 L bubble-column reactor with geometric relation height to diameter of 5 was used for the study of the kinetics of diesel oil consumption. Operation conditions and culture composition were the same used in the acclimation steps.

Kinetic of emulsified activity

The Emulsifying activity (EA) was determined with samples (5mL) taken at different times from reactors used in the acclimatization periods. Samples were centrifuged at 12 000 rpm at 4°C for 25 minutes, then 100 µL of supernatant (free cell), 2.6 mL of buffer TRIS-HCl (20 mM, pH 7) and $MgSO_4$ (10 mM) were added. 100 µL of mixture containing 2-phenyl-naftaleno: diesel 1:1 (v/v) were added immediately. Finally, the samples were sonicated for 5 minutes in order to form an emulsion, then said samples were allowed to stand for 24 hours; optical density was measured at 600 nm [24]. One Emulsifier Unit (EU mL^{-1}) was defined with a 0.1 change in absorbance units at 600 nm under assay conditions using diesel as a reference.

Volatile solids

In order to determine biomass production in the cultures (cycles 1 and 2, bubble-column), total solids were determined in reactors. The determination was done by the gravimetric method; 5 mL samples were homogenized and placed in porcelain capsules previously set to constant weight. The samples were placed in the oven at 100-105°C for 1 hour. The samples were calcined in a muffle at 500°C for 20 min. The total solid content was determined by the weight difference of the samples before and after calcination.

Diesel degradation

Gas chromatography (Varian model 3900, USA) with a flame ionization at 300˚C, a DB-Petro narrow-bore column (30×0.00025 m; J&W Scientific), and helium as the carrier gas detector was used to detect and quantify residual diesel in samples taken in the bubble column reactor. Commercial diesel was used as a standard reference.

RESULTS AND DISCUSSION

Diesel degradation

Fig. 1 shows that in the bubble column reactor, approximately 93.84% of the diesel was consumed using a sole source of carbon. The concentration of diesel was reduced from 13 to 0.8 g/L with the reactor operating for 15 days. However, it is important to note that by the third day of cultivation it had consumed 57.69 % of the diesel (until 5.5 g/L) at an average speed of 2.5 g / L day, and in the next 12 days, 36.15% of the diesel was consumed (until 0.8 g/L) at an average speed of 0.4 g / L day. As seen in these results, 93.84% of the diesel was consumed in the bubble-column reactor after 15 days of culture. The percentage of degradation of diesel reported in this research was higher than reported by *Ganesh* and *Lin* [5]. These authors report that the maximum level of diesel degradation was 80% using microorganism isolated (pure culture) from contaminated soil but, is important to point out that the maximum level of diesel degradation reported by these authors was achieved in a supplemented medium with 1% of glucose and an initial diesel concentration of 10 g/L. Diesel degradation rates and microbial cell number,

increases with an increase in glucose composition, having a positive effect, with an increase in growth of the isolates thus leading to significantly ($p < 0.05$) higher percentages of diesel degradation and greater emulsification activity according to [5].

On the other hand, it is also important to note that the maximum diesel degradation time reported in this study was five days less than the time reported by *Ganesh* and *Lin* (20 days) [5]. *Tan-Chen et al.* [6] reported 40-45% of diesel degradation in a shake flask reactor with a bacterial strain *Bacillus subtilis* BCRC16048, *Comamonas testosterone* CC-CF3, and *Sphingomonas yanoikuyae* CC-CG22, in 3 days of culture, which is lower than that reported in this study for the three days of culture; they report that the addition of 10% (v/v) EPS led to a 100-fold increase in viable cell counts and a 40% decrease in residual diesel.

Some researchers have shown that microorganisms isolated from hydrocarbon polluted soil are capable of degrading the contaminants by using it as a single source of carbon and energy or increasing their solubility with emulsifiers [5, 6]. In contrast, *Michaud et al.* [25] used two psychrotrophic bacterial strains isolated from Antarctic seawater (isolated from an unpolluted site) for diesel degradation. The results of this research show that two marine strains (E28 and E60) are able to degrade 86% and 89 % of the diesel (10 g/L) after 60 days of culture, which indicates that the microorganisms isolated from unpolluted sites have the capacity to degrade diesel [25]. However, it is important to note that the time required (60 days) to reach the levels of degradation reported (86 and 90 %) were much higher than those reported in this paper (15days). This demonstrates the importance of using microorganisms isolated from polluted soils[5, 6, 26], although the microorganisms isolated from unpolluted soil had the ability to use the hydrocarbon as a source of carbon and nitrogen, the process may require more time (75% more) [25]. These results indicate that polluted sites harbor a vast microbial population with the ability to metabolize hydrocarbons.

Emulsified activity

The microbial consortium used in this work showed the ability to produce emulsifying agents during cultivation in the bubble column (Fig 2). As a shown Figs. 2 and 3 the maximum emulsifier activity was

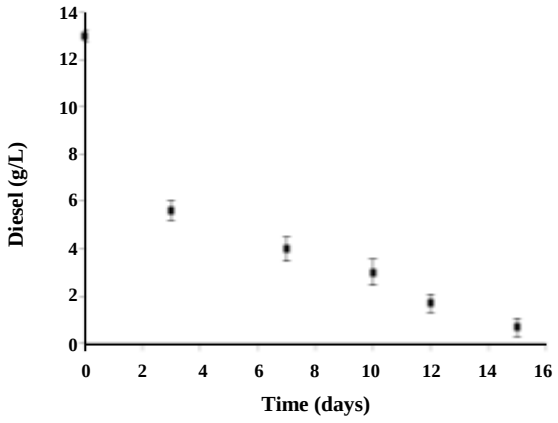

Fig. 1: Kinetic of diesel degradation in the Bubble-column.

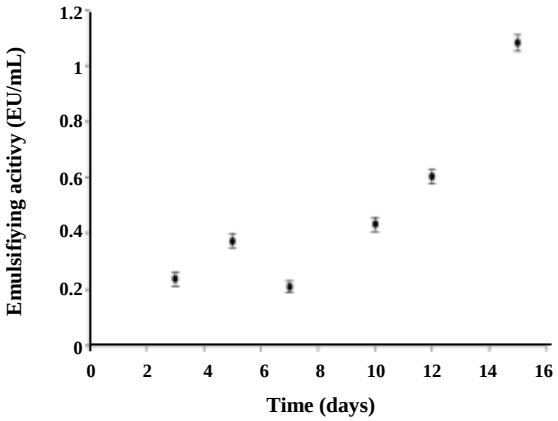

Fig. 2: Bioemulsifying activity produced by a consortium isolated from polluted soil using a bubble-column reactor.

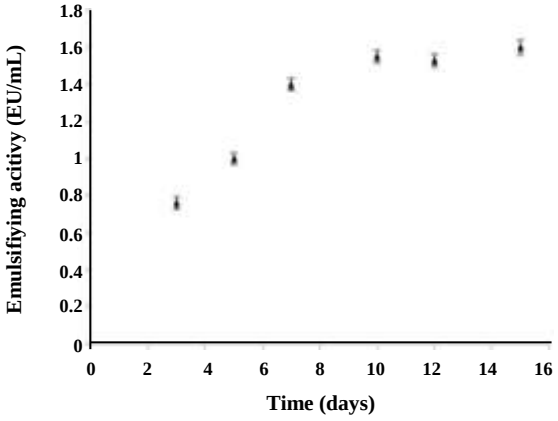

Fig. 3: Kinetics of volatile solids generated by a consortium isolated from polluted soil using a bubble-column reactor.

presented during the stationary growth phase, consistent with the results reported by some authors [17]. In Fig 1, after most of the diesel was consumed (day 8) bioemulsifier activity increases due to the carbon source is scarce, in comparison with previous days, and microorganism try to obtain better access to carbon source in a more soluble form. The production of bioemulsifiers can be induced by the addition of hydrocarbons or oils, it occurs by degradation of hydrocarbons that are utilized as nutrients by bacteria in limited nutrients condition [17]. In concordance with the literature [16, 27], the emulsifiers' maximum activity (Fig 2) was observed when the diesel was degraded almost totally (Fig 1) due to the low concentration of diesel generate more bioemulsifier production. The production of emulsifiers is very important in treatments for the degradation of hydrocarbons because the presence of these molecules may increase solubility or dispersion of substrates, in this work the rate of diesel degradation was in concordance with this asseveration [6]. The bioemulsifiers reported in this research can be used for remediation of contaminated soils. The biotechnological processes are able to remediate contaminated sites and are based on the use of microbial consortia to degrade hydrocarbons [23, 27, 28] but one of the highest cost in the process is microbial inoculum. The use of native biomass [23], as this article consider, could contribute to reducing economical cost. This kind of pneumatic reactors shows promise possibilities in order to design by mathematical models and scale-up [28] with environmental purposes [29].

CONCLUSION

Hydrocarbonclastic consortium isolated from polluted soil has the metabolic tools for diesel degradation (as a single carbon and energy source), also has the capacity to produce bioemulsifiers in a bubble-column reactor. Microbial consortium and bioemulsifiers produced in this research have the potential to use them in cleanup processes of polluted soil with hydrocarbons such as diesel.

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REFERENCES

[1] Obayori O.S., Ilori M.O., Adebusoye S.A., Oyetibo G.O., Omotayo A.E., Amund O.O, Degradation of Hydrocarbons and Biosurfactant Production by Pseudomonas sp. Strain LP1, *World. J. Microb. Biot.*, **25**(9):1615-1623 (2009).

[2] Okoh A.I., Biodegradation Alternative in the Cleanup of Petroleum Hydrocarbon Pollutants, *Biotechnol. Mol. Biol. Rev.*, **1**(2): 38-50 (2006).

[3] Hong S.H., Ryu H., Kim J., Cho K.S., Rhizoremediation of Diesel-Contaminated Soil Using the Plant Growth-Promoting Rhizobacterium Gordonia sp. S2RP-17, *Biodegradation.*, **22**: 593-601 (2011).

[4] Harrison A.B., Ripleya M.B., Dartb R.K., Bettsa W.B., Wilsona A.J., Effect of Protein Hydrolysate on the Degradation of Diesel Fuel in Soil, *Enzyme. Microb. Tech.*, **26**:388-393(2000).

[5] Ganesh A., Lin J., Diesel Degradation and Biosurfactant Production by Gram-Positive Isolates, *Afr. J. Biotechnol.*, **8**(21): 5847-5854(2009).

[6] Ta-Chen L., Chang J.S., Young C.C., Exopolysaccharides Produced by Gordonia Alkanivorans Enhance Bacterial Degradation Activity for Diesel, *Biotechnol Lett*, **30**:1201-1206(2008).

[7] Bento F.M., de Oliveira C.F.A., Okeke B.C., Frankenberger W.T., Diversity of Biosurfactant Producing Microorganisms Isolated from Soils Contaminated with Diesel Oil, *Microbiol. Res.*, **160**(3):249-255(2005).

[8] Kumar M., Leon V., De Sisto M.A., Ilzins O.A., Enhancement of Oil Degradation by co-culture of Hydrocarbon Degrading and Biosurfactant Producing Bacteria, *Pol. J. Microbiol.*, **55**(2):139-146(2006).

[9] Thavasi R., Subramanyam N.M., Jayalakshmi S., Balasubramanian T., Ibrahim M.B., Biosurfactant Production by Azotobacter Chroococcum Isolated from the Marine Environment, *Mar. Biotechnol.* **11**(5):551-556(2009).

[10] Ron E.Z., Rosenberg E., Biosurfactants and Oil Bioremediation, *Curr. Opin. Biotech.*, **13**: 249-252(2002).

[11] Venosa A.D., Zhu X., Biodegradation of Crude Oil Contaminating Marine Shorelines and Freshwater Wetlands, *Spill Sci Technol B*, **8**:163-178(2003).

[12] Pornsunthorntawee O., Wongpanit P., Chavadej S., Abe M., Rujiravanit R., Structural and Physiochemical Characterization of Crude Biosurfactant Produced by Pseudomonas Aeruginoa SP4 Isolated from Petroleum-Contaminated Soil, Biores. *Technol.* **99**:1589-1595(2008).

[13] Thavasi R., Jayalakshmi S., Balasubramanian T., Ibrahim M.B., Production and Characterization of a Glycolipid Biosurfactant from Bacillus Megaterium Using Economically Cheaper Sources, *World. J. Microbiol. Biotechnol.*, **24**(7):917-925(2008).

[14] Kumar M., Leon V., Materano A.D.S., Selvin J., Anto T.T., Rajeetha R.T., Natarajaseenivasan K., Biosurfactant Production and Hydrocarbon Degradation by Halotolerant and Thermotolerant Pseudomonas sp., *World. J. Microbiol. Biotechnol.*, **24**(7):1047-1057(2008).

[15] Rahman K.S.M., Banat I.M., Thahira J., Thayumanavan T., Lakshmanaperumalsamy P., Bioremediation of Gasoline Contaminated soil by a Bacterial Consortium Amended with Poultry Litter, Coir Pith, and Rhamnolipid Biosurfactant, *Biores. Technol.*, **81**(1):25-32(2002).

[16] Chandran P., Das N., Biosurfactant Production and Diesel Oil Degradation by Yeast Species Trichosporon Asahii Isolated from Petroleum Hydrocarbon Contaminated Soil, *Int J Eng Sci Technol*, **2**(12):6942-6953 (2010).

[17] Kokare C.R., Kadam S.S., Mahadik K.R., Chopade B.A., Studies on bioemulsifier Production from Marine Streptomyces sp. S1., *Ind. J. Biotechnol.*, **6**(1): 78-(2007)

[18] Margesin R., Labbe D., Schinner F., Greer C.W., Whyte L.G., Characterization of Hydrocarbon-Degrading Microbial Populations in contaminated and Pristine Alpine Soils, *Appl. Environ. Microbiol.* **69**:3085-3092(2003).

[19] Kasai Y., Takahata Y., Hoaki T., Watanabe K., Physiological and Molecular Characterization of a Microbial Community Established in Unsaturated, Petroleum-Contaminated Soil, *Environ. Microbiol.* **7**: 806-818(2005)

[20] Kaplan C.W., Kitts C.L., Bacterial Succession in a Petroleum Land Treatment Unit, *Appl. Environ. Microbiol.*, **70**:1777-1786 (2004).

[21] Röling W.F., Milner M.G., Jones D.M., Fratepietro F., Swannell R.P., Daniel F., Bacterial Community Dynamics and Hydrocarbon Degradation During a Fieldscale Evaluation of Bioremediation on a Mudflat Beach Contaminated with Buried Oil, *Appl. Environ. Microbiol.* **70**:2603-2613(2004).

[22] Yazdian F., Shojaosadati S.A., Nosrati M., Hajiabbas P., Khosravi K.M., On-Line Measurement of Dissolved Methane Concentration During Methane Fermentation in a Loop Bioreactor, *Iran. J. Chem. Chem. Eng. (IJCCE)*, **28**(4): 85-93(2009).

[23] Lizardi-Jiménez M. A., López Sánchez E. Y., Gómez-de-Jesús, A., García-Cruz N.U., Hernández-Flores C.I., Sinkhole-Native Oil-Degrading Microbial Consortium for Hydrocarbon- Polluted Seawater Remediation, *CLEAN–Soil, Air, Water*, **44**(8): 1046-1050 (2016)

[24] Toren A., Navon-Venezia S., Ron E. Z., Rosenberg, E., Emulsifying Activities of Purified Alasan Proteins from Acinetobacter Radioresistens KA53, *Appl. Environ. Microb*. **67**(3):1102-1106 (2001).

[25] Michaud L., Giudice A.L., Saitta M., De Domenico M., Bruni V., The Biodegradation Efficiency on Diesel Oil by Two Psychrotrophic Antarctic Marine Bacteria During a Two-Month-Long Eperiment, *Mar. Poll. B.*, **49**(5):405-409(2004).

[26] Singh C., Lin J., Isolation and Characterization of Diesel Oil Degrading Indigenous Microrganisms in Kwazulu-Natal, South Africa, *Afr. J. Biotechnol.* 7(12): - (2008).

[27] Angeles O., Medina-Moreno S. A., Jiménez-González A., Coreño A., Lizardi-Jiménez M. A., Predominant Mode of Diesel Uptake: Direct Interfacial Versus Emulsification in Multiphase Bioreactor, *Chem. Eng. Sci*. 165: 108-112 (2017).

[28] Denis B., Pérez O. A., Lizardi-Jiménez, M. A., Dutta, A., Numerical Evaluation of Direct Interfacial Uptake by a Microbial Consortium in an Airlift Bioreactor, *Int. Biodeter. Biodegr*. 119: 542-551 (2017).

[29] Shirdam R., Daryabeigi Zand A., Mehrdadi N. Removal of Total Petroleum Hydrocarbons (TPHs) from Oil-Polluted Soil in Iran, *Iran. J. Chem. Chem. Eng. (IJCCE)*, **28**(4): 105-113 (2009).