African Journal of Microbiology Research Vol. 6(2), pp. 393-402, 16 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1155
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Production of none germinate spore ghost from a novel marine *Bacillus* with thermostable laccase activity

Afrouzossadat Hosseini Abari*, Giti Emtiazi and Rasoul Roghanian

Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran 8174673441, I.R. Iran.

Accepted 11 November, 2011

Laccases are effective biocatalysts for various biotechnological applications. Treatment of effluents containing phenol components such as toluene using fungal laccases is usually limited to the acid to neutral pH range and moderate temperatures. In this research, several *Bacilli sp.,* were isolated from marine environment in toluene enriched media in purpose to obtain high laccase activities in spore forming bacteria. Among all the identified isolates, *Bacillus sp*. XJT-7 removed more toluene in contrast with other strains. The isolated *Bacillus* grew on toluene as the sole source of carbon and energy with maximum laccase activity and compared with *Bacillus sphaericus* (ATCC: CCM2177*)* as a standard strain which has laccase activity without any growth on toluene. Laccase activities in spore ghosts which mislay their germination power by heat shock have been investigated. The results showed that the vegetative cell free spore ghosts had thermostable laccase activities and remained active at 100°C shock. These heated spores (spore ghosts) are useful in biodegradation due to a high laccase activity. The image of Atomic Force Microscopy (AFM) showed that the spore ghosts have 50 nm pores which are good for drug delivery and drug synthesis with laccase activity without any chances for having spore germination.

Key words: *Bacillus* sp., spore ghost, Laccase, atomic force microscopy (AFM).

INTRODUCTION

Laccases are blue multicopper polyphenol oxidases (PPO) which couple the oxidation of various substrates with the reduction of O_2 to H_2O (Leonowicz et al., 2001; Yaropolov et al., 1994). They are naturally distributed in plants, fungi and prokaryotes (Bonugli et al., 2010; Claus and Filip, 1997; Harvey and Walker, 1999) such as *Azospirillum lipoferum* (Diamantidis et al., 2000), *Marinomonas mediterranea* (Sanchez-Amat et al., 2001), *Sinorhizobium meliloti* (Rosconi et al., 2005) and *Pseudomonas desmolyticum* (Kalme et al., 2009). Laccase-like activity has also been found in other bacteria, for example, CopA protein from *Pseudomonas syringae* (Cha and Cooksey, 1991) and PcoA protein from *Escherichia coli* (Kim et al., 2001). Recently scientists determined spore-bound laccases which are stable at high temperatures and pH values but these spores have germination power and may create some problems. The best-studied for bacterial laccase is the CotA, the endospore coat component of *Bacillus subtilis*. The cotA gene codes for a 65-kDa protein belonging to the outer spore coat. CotA participates in the biosynthesis of the brown spore pigment and seems to be responsible for most of the protection afforded by the spore coat against ultraviolet (UV) light and hydrogen peroxide (Hullo et al., 2001; Riesenman and Nicholson, 2000). CotA protein displays similarities with multi-copper oxidases and exhibits a higher thermal stability with a half-life of about 2 h at 80°C and optimum temperature 75°C (Hirose et al., 2003). Laccase is used to make different drugs like triazolo (benzo) cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, dimerized vindoline and iodine

*Corresponding author. E-mail: afrouz_hosseini1985@yahoo.com. Tel: +983117932457. Fax: +983117932456.

(Leonowicz et al., 2001; Molino et al., 2004; Stahl et al., 2002). It is also used for the treatment of aceruloplasminemia (Harris et al., 2004), for bioremediation and biodegradation of phenolic and polyaromatic hydrocarbon compounds (Hublik and Schinner, 2000).

In the past decade, more attention has been focused on the applications of nanostructure materials because of their benefits as biosensors, bioreporters and biofuel cells. Nanotechnology laccases can be applied as bioreporters such as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assay and as biosensors for immunoassays, and for the determination of glucose, aromatic amines and phenolic compounds (Bauer et al., 1999; Simkus et al., 1996; Kunamneni et al., 2008). In addition to biosensors, immobilized laccases on the cathode biofuel cells can be used for small transmitter systems. The laccase-based miniature biological fuel cell is of particular interest for many medical applications calling for a power source implanted in the human body (Heller et al., 2008). The immobilized spore laccases can also be used for the continuous biotransformation of any laccase substrate (Held et al., 2005). Due to high temperature and pH stability, these spores are compatible with industrial processes. For this purpose, *Bacillus* strain isolated on toluene agar, then toluene biodegradation and laccase activity were studied in spores and spore ghosts. In this study, for the first time spore ghost (inactive spore) from marine bacteria has been presented as an immobilized laccase nanoparticle. Also AFM was used to compare the structure of the spore ghost with the active spore (with germination capability).

MATERIALS AND METHODS

Media preparation

For the isolation of marine bacteria, toluene-contaminated seawater samples were collected from three different sites in the Persian Gulf and two sites in the Caspian Sea and the Gulf of Oman during spring. Seawater samples were collected from a depth of 15 cm in sterile 100 ml bottles and transported on ice to a laboratory for isolation.

Isolation of bacteria

A liquid mineral medium (MM) was prepared isolation of toluene degrading bacteria. This medium contained (gl^{-1}) KH_2PO_4 4, Na_2HPO_4 4, NH_4Cl 2, $MgSO_4$ 0.2, $CaCl_2$ 0.001, $CuSO_4$ 0.001 and $FeCl_3$ 0.001 in 1000 ml distillated water and pH 6.8. About 5 ml seawater and 1ml wastewater samples were added to 50 ml of sterile mineral medium in 250 ml flasks. Toluene was added to the sterile mineral medium by 1% (v/v). The flasks were incubated at 28°C. After one week of enrichment, 1ml culture was transferred into a fresh medium and further incubated. After four subcultures, an appropriate dilution of the culture was spread onto the toluene agar (mineral medium with toluene and agar) plates. Phenotypically different colonies obtained from the plates were transferred to fresh mineral medium with and without toluene to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated and the marine spore-forming bacteria which showed significant growth on toluene were stored for further characterization (Wang et al., 2008).

Identification of isolates

Isolated strains were biochemically analyzed for Gram staining, acid-fast staining, the catalase test, the oxidase test, oxygen requirement, motility, the ability to grow on different carbon sources and in the presence of some inhibitors according to the standards of microbial identification (Slepecky and Hemphill, 2006).

Growth and toluene removal assay

The growth rates of the isolates were routinely assessed indirectly by a turbidity measurement as (O.D. at 600 nm) in a UV visible spectrophotometer (Shimadzu UV-160, Japan). The toluene removal assay was carried out by dissolving the residual toluene of the aforementioned medium in 1 ml n-hexane and reading the optical density of the toluene against a blank at 261 nm wavelength. The control was a sterile medium (Wang et al., 2008).

Spore preparation

The marine spore-forming species were grown in the nutrient agar and toluene agar (MM agar medium with toluene as a carbon source) and incubated in 28°C for 24 h. Then these plates were transferred to an incubator set at a temperature level of 37°C for five days. After incubation the plates were stored in a refrigerator set at a temperature level of 4°C for two weeks for the enhancement of the spores.

Removal of vegetative cells form bacterial spore

After maximum sporulation, the spores and remaining vegetative cells were removed from the growth media by heating shock (for *Bacillus* species 80°C, 10 min and for *Sporosarcina* 70°C 10 min) and centrifugation at 2000 rpm for 30 min in the cold. The supernatant having a lot of spores was tested with a microscope. The final stock spore suspension contained approximately 10^7 spores/ml as determined by direct aerobic colony counts on nutrient agar (NA) (Sterling and Williams, 1958).

Spore ghost preparation

For spore ghost production, strains were heated and then filtered by millipore filtration. After filtration the filters were washed by sterile double distillated water to isolate the ghost and stained with malachite green as the main color and safranine as the background.

Laccase activity

Laccase activity was measured in 1ml reaction measure containing 75 mM catechol as substrate in 50 mM sodium phosphate buffer, pH5 and 200 µl of culture fluid. The progress of the reaction was monitored at 440 nm for 10 min. One unit of laccase activity was defined as a change in A_{440} of 1ml in 1min (Ruttimann et al., 1992).

Atomic force microscopy

All imaging was done by scanning probe microscope (Dualscope/

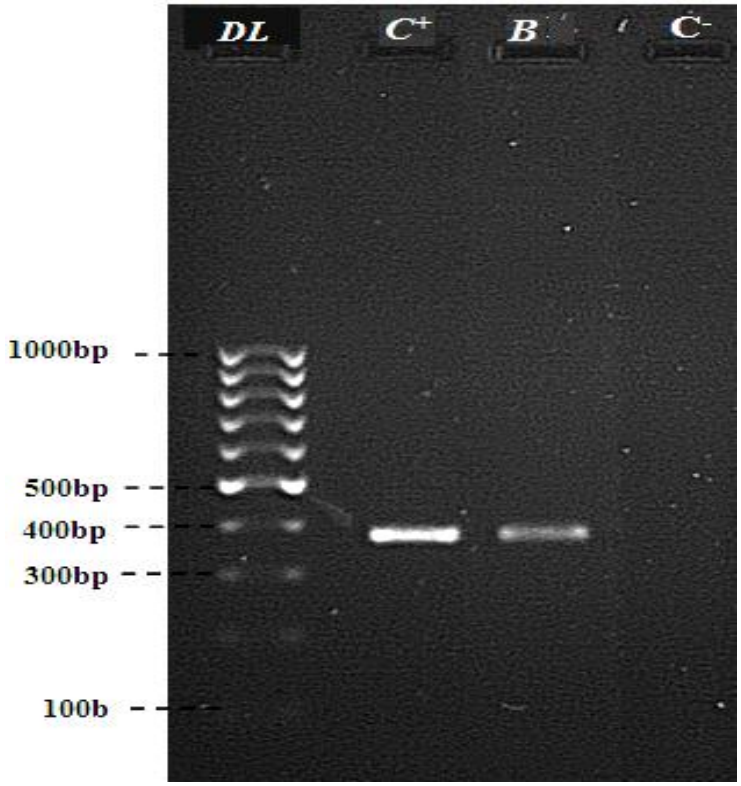

Figure 1. Electrophoresis gel for the PCR products. DL: 100 bp DNA ladder, C^+: Positive control*: B. sphaericus* ATCC: CCM2177, C^-: Negative control, B: *Bacillus* sp. XJT-7.

Rasterscope C26, DME, Denmark) with DS 95-50-E scanner. Silicon probes of resonant frequency 150 to 190 kHz were used (Model Mikromasch NSC16). New cantilever aluminum coating was used for each sample to prevent cross-contamination. Seven to ten spores of each species, selected randomly, were imaged by AFM. Both topography and phase images were captured.

Genotypic characterization

The best marine *Bacillus* with high laccase activity was selected for genotypic characterization. Chromosomal deoxyribonucleic acid (DNA) isolation was made by the heating method 100°C for 10 s-from cells grown overnight at 28°C in TSB (Tryptone Soya Broth). DNA concentration was quantified by UV Biophotometer at 260 nm (Eppendorf AG: 22331 Hamburg). 16s ribosomal deoxyribonucleic acid (rDNA) genes were amplified with DG74-AGGAGGTGATCCAACCGCA as a forward primer and RW01-AACTGGAGGAAGGTGGGGAT as a backward primer (Bickley et al., 1996). Polymerase chain reaction products were separated by agarose gel electrophoresis. Purification and sequencing was performed by Eurofins MWG Operon's sequencing service, Germany.

RESULTS

Isolation and characterization of toluene utilizing bacteria

Twenty toluene-degrading bacterial strains were isolated from the seawater of the Persian Gulf and Caspian Sea. Among them three Gram-positive rods, one Gram-positive coccus was selected for more study. The Gram positive, spore-forming, oxidase- negative and catalase-positive rods which produce convex orange colonies were identi-fied as *Bacillus amyloliquefaciens*. The Gram-positive coccus, which had aerobic growth and which was capable of nitrate reduction, oxidase and catalase positive and capable of endospore forming, was identified as *Sporosarcina halophila*. The Gram-positive spore-forming, oxidase negative catalase-positive rods which produce slime white colonies were identified as *Bacillus firmus*. The 16s rDNA sequence analyses for one strain that with highest laccase activity showed that this marine *Bacillus* was close to *Bacillus sp.*XJT-7(GU269615.1) (98%) and is constituted by Gram-positive, spore-forming, oxidase negative and catalase-positive rods which produce slime black colonies. The electrophoresis gel is shown in Figure 1.

Growth rate and toluene removal by isolates

After 24 h, strains grown in the mineral medium by toluene as the carbon and energy source reduced the toluene (Figure 2). The results showed that *Bacillus* sp.

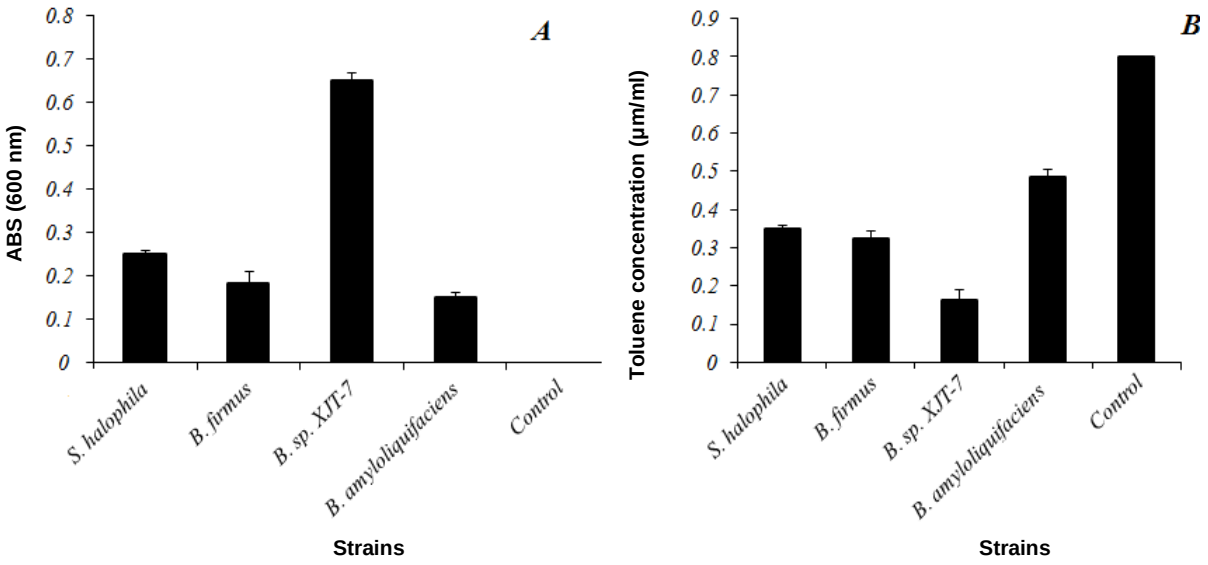

Figure 2. A The growth rates of isolates in toluene after 24 h; **B** Analyses of toluene consumption by strains, assayed with UV spectroscopy in 261 nm after 24 h (the averages of three replications have been shown). Control was the sterile mineral medium with toluene.

Table 1. The effect of temperature for the inactivation of spore then ghost production.

Strains	Temperature (°C)	Time (min)
S. halophila	80	10
B. sphaericus	100	10
B. sp. XJT-7	100	10
B. firmus	100	30
B. amyloliquefaciens	100	30

XJT-7 had a high ability of growing on toluene as the only source of carbon and energy and removed toluene by 65%.

Checking of spore ghost preparation

The inactivation of spores from different strains occurred at different temperatures (Table 1). The specific temperature and time was identified by lack of growth on nutrient agar after heating. As is shown, the spore of *B. sphaericus* is resistant to 100°C for 10 min, however, some strains are resistant to 100°C for 30 min. Spore staining showed that the spores had been damaged and with malachite green staining (Wirtz- Conklin method) had turned red. Therefore, we supposed that spore ghosts have pores on their surface through which malachite green is washed from the core.

Laccase assay in vegetative cells

The supernatant of all strains grown on toluene showed that the vegetative cells have laccase activity. In this situation, induction with toluene was more effective than without toluene (Figure 3).

Laccase assay in spores and spore ghosts

The cell free spores were obtained by filtration and the laccase activities of the spores were determined and compared with those of *B. sphaericus*. The laccase activity had an additive process during eight days. The results are shown in Figure 4. Induction with toluene did not have any significant effect on laccase activity in most strains (Figure 5). The laccase activities of the cell free spore and the heated spore (ghost) are shown in Figure 6. As is shown *Bacillus* sp.XJT-7 significantly has more laccase activities when it was heated for 10 min.

Atomic force microscopy of the *Bacillus* spore and the *Bacillus* ghost

Bacillus spore surface morphology was imaged with AFM

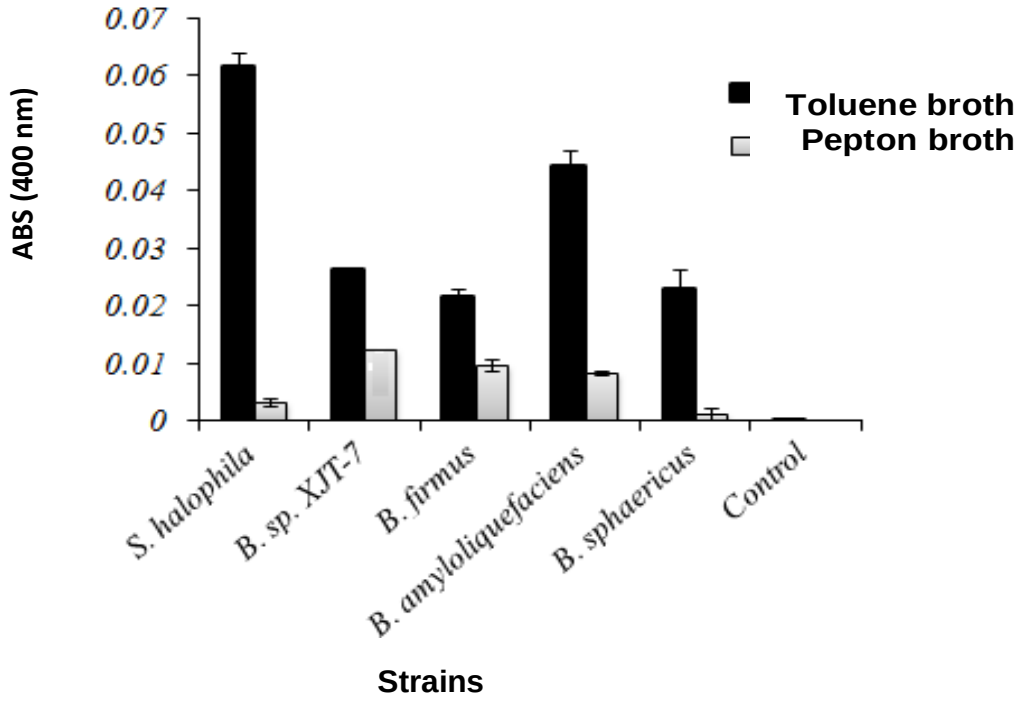

Figure 3. Comparison of laccase activity between vegetative cells in cell free supernatant from toluene and peptone broth (the averages of three replications have been shown). Control was without bacterial inoculation.

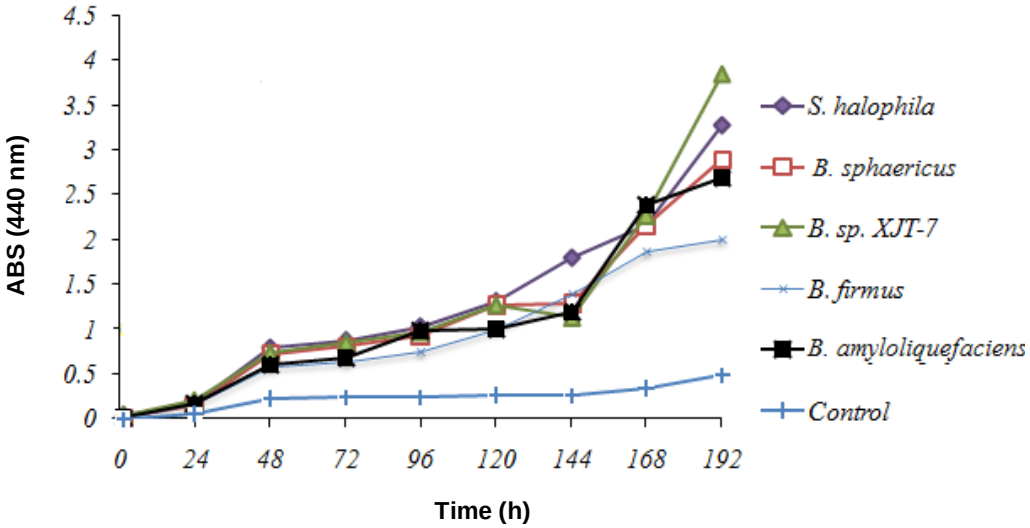

Figure 4. The effect of time on laccase activities in the spores of different strains.

to determine if the characteristic surface features of the spore ghost could be used for drug syntheses, delivery and bioremediation without any chance of spore germination. For this purpose, spore was collected with millipore filter and was heated such that no live cell could be seen in the nutrient agar. The spore and spore ghost was imaged with AFM. The AFM images of the spores showed that there were no damages to the spores. Their size was 2*2.61 µm with a roughness of 36.7% and a surface of 243 nm with a smooth layer and they had laccase activities; however the spore ghosts were damaged and were smaller with a rough surface and had 50 nm pores (Table 2). Figures 7, 8 and 9 show AFM images of the spores and the surface topography and

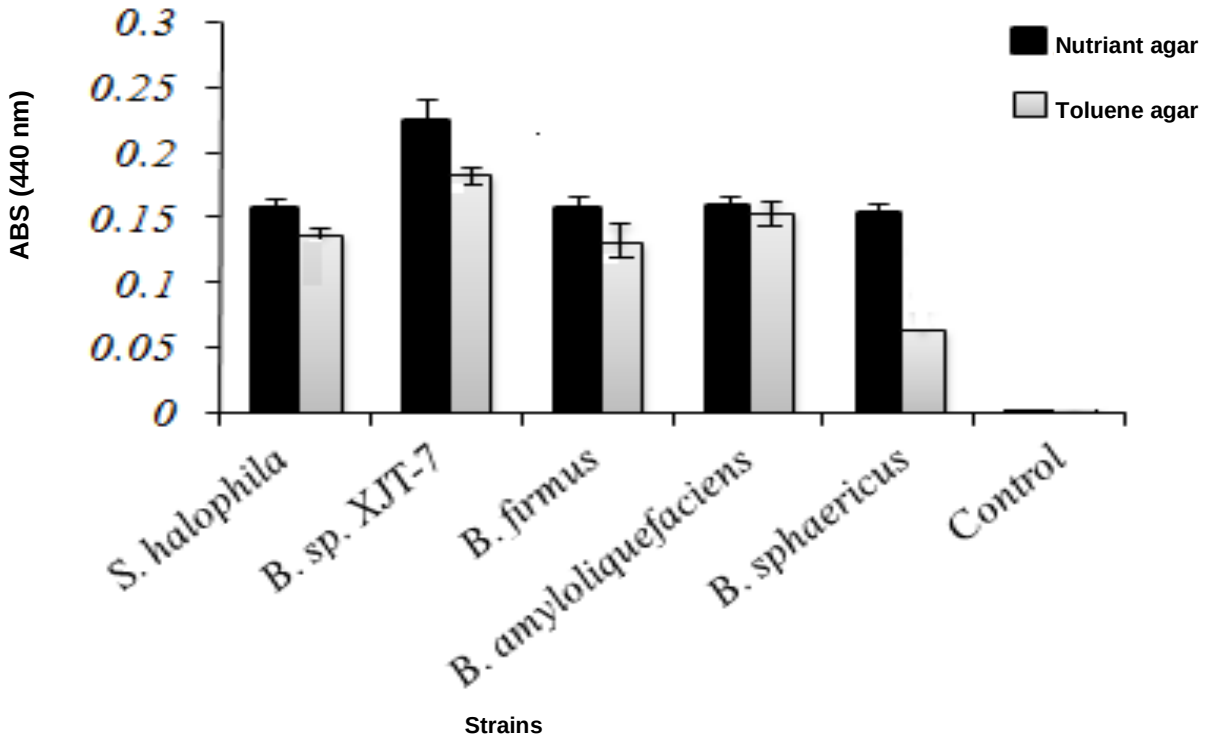

Figure 5. Comparison of laccase activity in spores obtained from nutrient and toluene agar (the averages of three replications have been shown). Control was sterile medium.

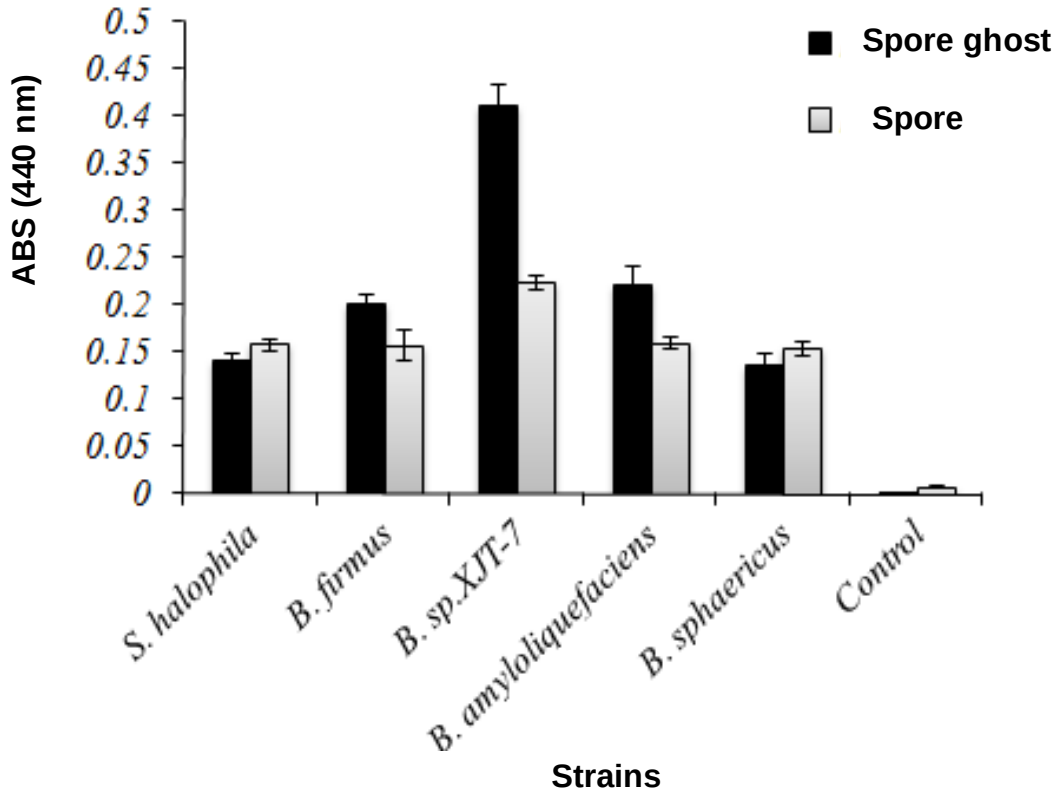

Figure 6. Comparison of laccase activity in spore ghost and spore obtained from nutrient agar (the averages of three replications have been shown). Control was sterile medium.

Table 2. AFM analysis of spore and spore ghost.

Properties	Spore	Spore ghost
Width (µm)	2	1
Height (µm)	2.61	1.26
Roughness (%)	36.7	78.2
Core's width (nm)	418.5	204
Core's height (nm)	780	394
Width of layers (nm)	243	100
Pore (nm)	-	42 to 50

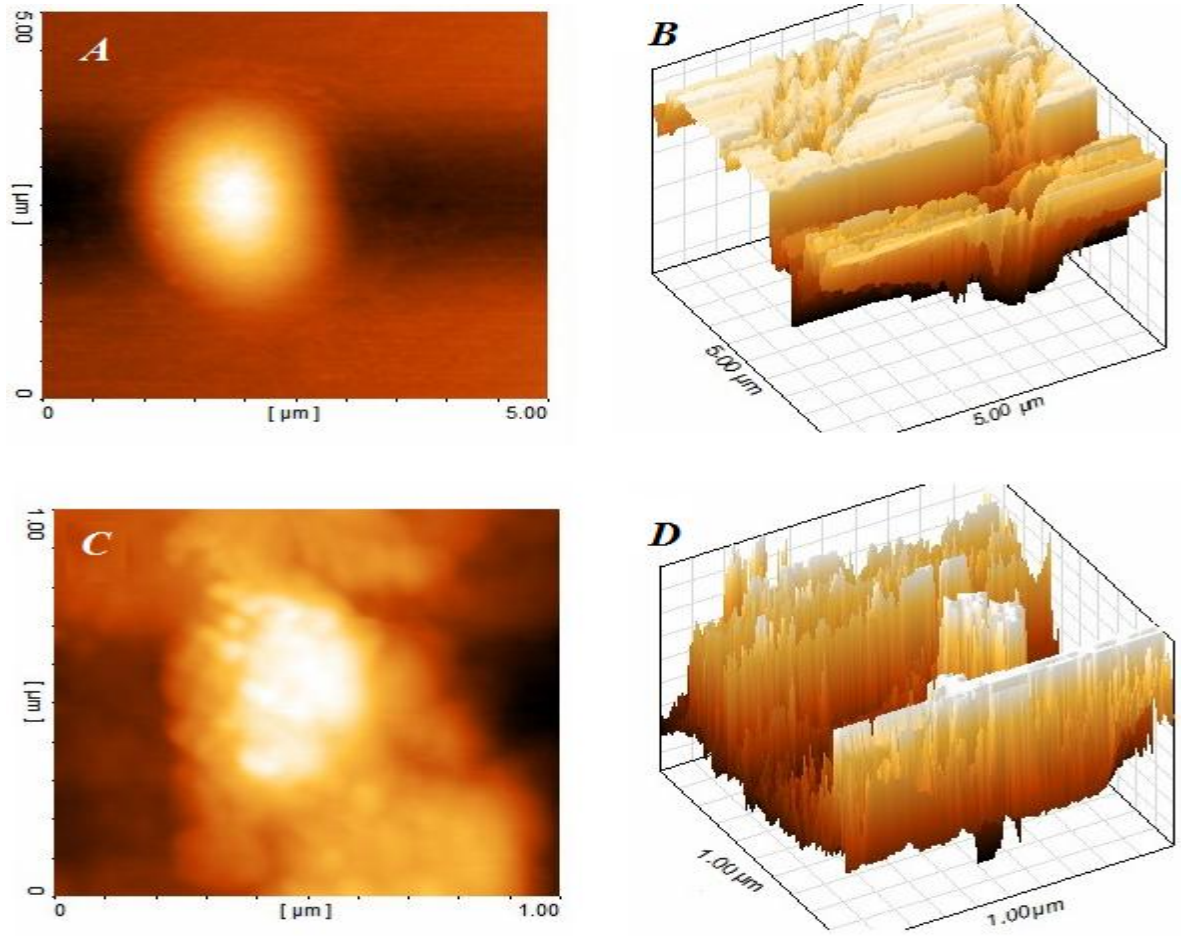

Figure 7. AFM images of *Bacillus sp. XJT-7* spore and spore ghost. A (spore 2*2.61um, topography data), B (Spore, 3 dimential phase data), C (spore ghost 1*1.26 um, topography data), D (Spore ghost, 3 dimential phase data).

surface roughness of heated and not heated spore.

DISCUSSION

The rate of laccase activity in spores and spore ghosts is more than that of vegetative cells and the laccase production in the spores was not different in induction and non induction media. Although toluene was not needed for the induction of laccase in the spores, induction with toluene had increased laccase activity in vegetative cells. Toluene biodegradation in vegetative cells may be under the impression of laccase activity (Hullo et al., 2001). Since laccase activity in spores depends on CotA protein (Kalme et al., 2009; Bauer et al., 1999) induction with toluene probably is not necessary. In this article, for the first time, laccase activity in *Bacillus sp.XJT-7* was studied; it was noticeably more than the standard strain,

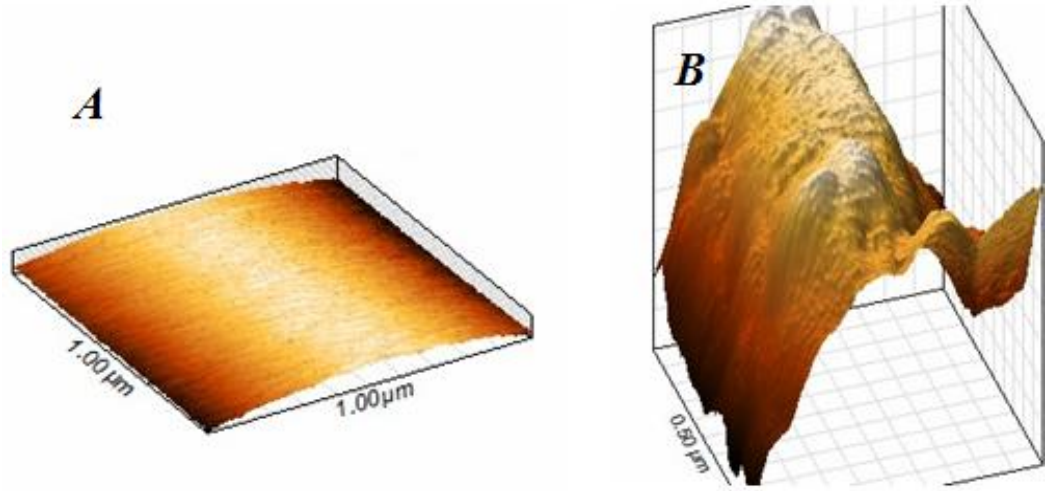

Figure 8. AFM images of surface topography data (3D). A (Spore), B (Spore ghost).

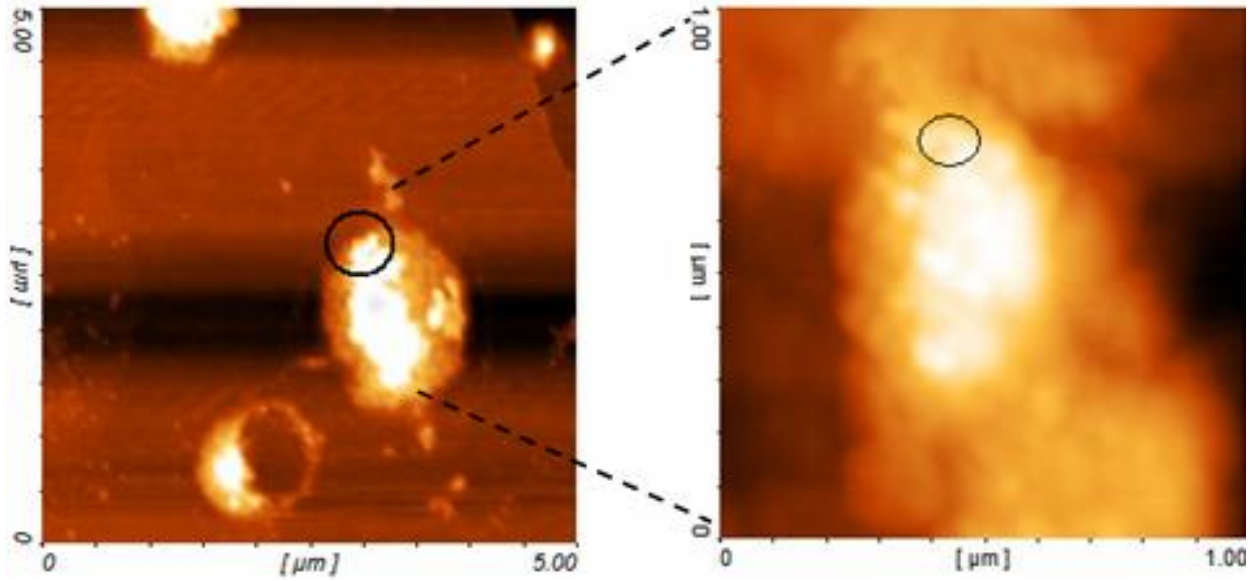

Figure 9. Spore ghost topography data. The diagonal induced pore is 50 nm.

B. sphaericus. Thus the spore and spore ghost of this novel strain, *Bacillus sp*.XJT-7, is a good candidate for industrial uses and bioremediation. Laccase activity in spores was progressively increased during eight days up to the consumption of all of the catechol. Fortunately, laccase activity in the spore ghosts did not decrease but showed significant increase in *Bacillus sp*. XJT-7 with a heat shock (Figure 5). Zolock et al. (2006) showed that AFM revealed that spore surface is rich with information for distinguishing the samples of different spores of closely related species (Zolock et al., 2006). Spore ghost surface was significantly different from spore surface. Its size was in the nano-scale. Due to the high laccase activity, we can claim that the spore ghost is the novel nanoparticle with active immobilized laccase.

The existences of 50 nm pores in the spore ghost were observed by AFM and are good candidates for drug or gene delivery. However the ghost was not alive, the laccase activity was significantly higher than that of the unheated spore (Figure 6). This nanoparticle is very usable in industry, drug synthesis and bioremediation. Also spore ghost is used as a biosensor and bioreporter in nanotechnology and this damaged spore acts as an empty capsule for remediation (with laccase activities) and textile industry is resistant to heat and high temperature (Hirose et al., 2003; Enguita et al., 2003),

the spore ghost is the best candidate to use. Laccases have been immobilized on chitosan and many nanometals (Hua et al., 2007; Qiu et al., 2009) but their producing processes are difficult and expensive on a large scale basis and these ways are not cheap. Since spore ghost preparation is inexpensive and easy, it is clear that the spore ghost, in connection with the use of laccase, has opened a novel interesting way for its applications. The systemic application of anticancer drugs often causes severe toxic side effects for drug production because of their toxicity. To reduce the undesired effects, advanced drug delivery systems are needed which are based on specific cell targeting vehicles (Paukner et al., 2004). Used bacterial ghosts from *Mannheimia haemolytica* for site-specific delivery of doxorubicin (DOX) to human colorectal adenocarcinoma cells (Caco-2) (Paukner et al., 2004). Bacterial ghosts are non-denatured envelopes of Gram-negative bacteria with fully intact surface structures for specific attachment to mammalian cells. To our best knowledge spore ghost is not used for drug delivery but bacterial spore is used as immobilized laccase enzyme. Therefore the spore ghost obtained from marine *Bacillus* is a good candidate for bioremediation and drug delivery. Also this small particle can immobilize lantibiotic for food preservative and can be used in nanotechnology.

ACKNOWLEDGEMENTS

We thank the University of Isfahan for financial support given to MS student for a training period in the Department of Biology and Microbiology.

REFERENCES

Bauer CG, Kuhn A, Gajovic N, Skorobogatko O, Holt PJ, Bruce NC, Makower A, Lowe CR, Scheller FW (1999). New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase. Fresenius. J. Anal. Chem., 364: 179-183.

Bickley J, Short JK, McDowell DG, Parkes HG (1996). Polymerase Chain Reaction (PCR) detection of *listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett. Appl. Microbiol., 22: 153-158.

Bonugli-Santos RC, Durrant LR, Silva Mda, Durães Sette L (2010). Production of laccase, manganese peroxidase and lignin peroxidase by Brazilian marine-derived fungi. Enzyme. Microbial. Technol., 46: 32–37.

Cha J, Cooksey DA (1991). Copper resistance in *Pseudomonas syringae* by periplasmic and outer membrane proteins. Proc. Natl. Acad. Sci. USA., 88: 8915–8919.

Claus H, Filip Z (1997). The evidence for a laccase-like enzyme activity in a *Bacillus sphaericus* strain. Microbiol Res., 152: 209–216.

Diamantidis G, Effosse A, Potier P, Bally R (2000). Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*. Soil. Biol. Biochem., 32: 919–927.

Enguita FJ, Martins LO, Henriques AO, Carrondo MA (2003). Crystal structure of a bacterial endospore coat component: a laccase with enhanced thermostability properties. J. Biol. Chem., 278: 19416-19425.

Harris ZL, Davis-Kaplan SR, Gitlin JD, Kaplan J (2004). A fungal multicopper oxidase restores iron homeostasis in aceruloplasminemia. Blood, 103: 4672-4673.

Harvey BM, Walker JRK (1999). Studies with plant laccases: I. Comparison of plant and fungal laccases. Biochem. Mol. Biol. Biophys., 3: 45–51.

Held C, Kandelbauer A, Schroeder M, Cavaco-Paulo A, Guebitz GM (2005). Biotransformation of phenolics with laccase containing bacterial spores. Environ. Chem. Lett., 3: 74–77.

Heller A, Mano N, Kim H, Zhang Y, Mao F, Chen T, Barton S (2008). Miniature biological fuel cell that is operational under physiological conditions, and associated devices and methods. Patent US 2008118782.

Hirose J, Nasu M, Yokoi H (2003). Reaction of substituted phenols with thermostable laccase bound to *Bacillus subtilis* spores. Biotechnol. Lett., 25: 1609–1612.

Hua F, Jun H, Liyun D, Mingtian LI, Zhao C (2007). Preparation of magnetic chitosan nanoparticles and immobilization of laccase. J. Wuhan. Univ. Technol. Mater. Sci. Edition, 24: 42-47.

Hublik G, Schinner F (2000). Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. Enzyme. Microbial. Technol., 27: 330–336.

Hullo MF, Moszer I, Danchin A, Verstraete IM (2001). CotA of *Bacillus subtilis* is a Copper-Dependent Laccase. J. Bacteriol., 183: 5426–5430.

Kalme S, Jadhav S, Jadhav M, Govindwar S (2009). Textile dye degrading laccase from *Pseudomonas desmolyticum* NCIM 2112. Enzyme. Microbial. Technol., 44: 65–71.

Kim C, Lorenz WW, Hoopes T, Dean JFD (2001). Oxidation of siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J. Bacteriol., 183: 4866–4875.

Kunamneni A, Plou FJ, Ballesteros A, Alcalde M (2008). Laccases and their applications: a patent review. Recent. Pat. Biotechnol., 2: 10-24.

Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wojtas-Wasilewska M, Matuszewska A, Hofrichter M, Wesenberg D, Rogalski J (2001). Fungal laccase: properties and activity on lignin. J. Basic. Microbiol., 41: 185–227.

Molino BF, Haydar SN, Yang Z, Michels PC, Hemenway MS, Rich JO, Khmelnitsky Y (2004). Preparation of novel cyclosporins. Patent WO2004082629 A2, 2004.

Paukner S, Kohl G, Lubitz W (2004). Bacterial ghosts as novel advanced drug delivery systems: antiproliferative activity of loaded doxorubicin in human Caco-2 cells. J. Control Release, 94: 63–74.

Qiu H, Xu C, Huang X, Ding Y, Qu Y, Gao P (2009). Immobilization of Laccase on Nanoporous Gold: Comparative Studies on the Immobilization Strategies and the Particle Size Effects. J. Phys. Chem. C., 113: 2521–2525.

Riesenman PJ, Nicholson WL (2000). Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Appl. Environ. Microbiol., 66: 620–626.

Rosconi F, Fraguas LF, Mart´ınez-Drets G, Castro-Sowinski S (2005). Purification and characterization of a periplasmic laccase produced by *Sinorhizobium meliloti.* Enzyme. Microbial. Technol., 36: 800–807.

Ruttimann C, Schweber E, Salas L, Cullen D, Vicuna R (1992). Lygninolytic enzymes of white rot basidiomycetes *Phlebia brevispora* and *Ceioporiopsis subvermispora*. Biotechnol. Appl. Biochem., 16: 64-76.

Sanchez-Amat A, Lucas-Elio P, Ferandez E, Garcia-Borron J C, Solano F (2001). Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from *Marinomonas mediterranea.* Biochim. Biophys. Acta., 1547: 104–116.

Simkus RA, Laurinavicius V, Boguslavsky L, Skotheim T, Tanenbaum S, Nakas JP, Slomczynski DJ (1996). Laccase containing sol-gel based optical biosensors. Anal. Lett., 29: 1907-1919.

Slepecky RA, Hemphill HE, (2006). The genus *Bacillus*, in: Dworkin M, Falkow S, Rosenberg E, K.H. Schleifer, E. Stackbrandt (eds), The Prokaryotes, third ed., Springer, New York, 4: 530-562.

Stahl P, Kissau L, Mazitschek R, Giannis A, Waldmann H (2002). Natural product derived receptor tyrosinkinase inhibitors: Identification of IGF1R-, Tie-2 and VEGFR3 inhibitors. Angew. Chem. Int. Ed., 41: 1174-1178.

Sterling SK, Williams OB (1958). Methods for removal of vegetative

cells from bacterial spore preparations. J. Bacteriol. ASM., 76: 332- 335.
Wang L, Qiao N, Sun F, Shao Z (2008). Isolation, gene detection and solvent tolerance of benzene, toluene and xylene degrading bacteria from nearshore surface water and Pacific Ocean sediment. Extremophiles., 12: 335-342.
Yaropolov AI, Skorobogat'ko OV, Vartanov SS, Varfolomeyev SD (1994). Laccase: Properties, catalytic mechanism, and applicability. Appl. Biochem. Biotechnol., 49: 257-280.
Zolock RA, Li G, Bleckmann C, Burggraf L, Fuller DC (2006). Atomic force microscopy of *Bacillus* spore surface morphology. Micron. 37: 363–369.