Silanization and sorption of crystalline CotA particles from highly oxidative *Bacillus sp.* LPG-1 spores on slide and perlite for oxidase activities

Afrouzossadat Hosseini Abari [a], Giti Emtiazi[1]* and Mohammad Rabbani Khorasgani[a]

[a] Department of Biology, Faculty of Science, University of Isfahan, Isfahan, IR Iran
**Corresponding author*: Giti Emtiazi, Department of Biology, Faculty of Science, University of Isfahan, Isfahan, IR Iran. Tel: + 98-3117932457, Fax: + 98-3117932456, e-*mail:* Emtiazi@yahoo.com

Abstract

Spores are the resting form of bacteria exhibiting resistance to extreme conditions so they are suitable particles for use in industry and elimination of hazardous material from environment. Bacterial spores have several enzymatic activities that the most of them are because of the crystalline CotA protein. Accordingly, the aim of this study was to detect enzymatic activities of different bacterial spore forms and compare them with immobilized spores on silanized glass slide and perlite granules. To determine enzyme activities of the spores, spectrophotometery methods were used. In this study for the first time, we analyzed catalase activity using an easy quantitative colorimetric method. Also, X-ray diffraction (XRD) technique was used to determine crystalline structure of CotA obtained from broken spore. Fourier transform infrared spectroscopy (FTIR) was applied as a rapid and easy method to differentiate spore and crystalline cotA particles. Crystalline CotA particles of *Bacillus* LPG-1 that was prepared by heating represented higher enzymatic activities (laccase, catalase, phenol oxidase) than the other forms of spore. Because of covalent attachment of spores on silanized glass slide and perlite, enzymatic activities of immobilized spores slightly decreased in comparison with free spores.

The results of the present study suggested that bacterial spores can be widely used in industry due to their significant enzyme activities. Although immobilization may lower enzyme activities of spores, capability of reuse them can compensate this weakness.

Keywords: Crystalline CotA particles, Enzyme activity; Fourier transform infrared spectroscopy; Perlite;; Silanized glass slide; X-ray diffraction technique

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Introduction

In the past, bacterial spores were mentioned as an inactive form but nowadays several enzymatic activities in the spores have been detected. Thus, scientists introduce bacterial spores as a "Biochemical Catalysts in Life and Industry" (Ruehle 1923). Some proteins locating on the spore coat have unique properties. The 65-kDa CotA protein is one of the abundant proteins in the outer coat layer that recently represented highly thermostable laccase-like activities (Martins *et al*. 2002) Laccases are polyphenol oxidases (Hullo *et al*. 2001), and belong to the multicopper oxidase family (Dube *et al*. 2008; Enguita *et al*. 2003). Despite laccase activity, the resistance of spores to hydrogen peroxide depends on the expression of cotA (Riesenman and Nicholson 2000). Spores with their oxidative activities can be used as particles for oxidation of pollutants and petroleum components in waste water and environment. Since the spores have particular properties including resistance to

dryness, UV radiation, salinity, variable pH and heat, it is suitable to be used in industry.

Enzyme immobilization on a solid support makes them reusable, more stable against thermal denaturation, and also protects them from irreversible inactivation (Balcao *et al*. 2001). Two solid surfaces that we used in this study were perlite and silanized glass slide. Perlites are amorphous aluminum silicates with high content of silica more than 70%. Commercially it is focused due to their thermal and mechanical stability, no-toxicity, and high resistance against microbial attacks and organic solvents (Bai *et al*. 2006; Sakaguchi *et al*. 2005; Tischerw 1999). Silane derivatives act like a glue and are used as coupling agents to adhere glass slides to a polymer matrix. In the other words, silane coats the glass slides to create better adhesion to the polymer chain (Labit *et al.* 2008).

In the present study for the first time, spores were immobilized on the solid surfaces of perlite and silanized glass slide, then their catalase, laccase, and phenol oxidase activities were assayed and compared with the free spores. Before and after immobilization on silanized slide, the structure of spore was studied by X-ray diffraction technique. Also, Fourier transform infrared spectroscopy as a rapidly monitoring tool was applied in the region 4000-400 cm^{-1}(Ammann and Brandl 2011; Subramanian *et al*. 2005) to study the spores, crystalline CotA structures and their attachments on perlite. Here, we have shown that both the spore and the crystalline CotA particles are remarkably heat stable enzymes.

Experimental

Materials and Methods

Bacterial strains, growth conditions and spore preparation

Bacillus LPG-1 was isolated from our previous study in toluene enriched medium and *Bacillus sphaericus* CCM2177 was used as a standard strain (Hosseini Abari *et al*. 2012). Both of them were inoculated into Difco Sporulation Medium (DSM) containing 0.8 % (w/v) Difco Nutrient

Broth, 0.1% KCl, 0.025% $MgSO_4 \cdot 7H_2O$, 1 mM Ca $(NO_3)_2$, 0.01 mM $MnCl_2$ and 0.01 mM $FeSO_4$ pH 7, and incubated at 28°C for 24 h on a shaker with 150 rpm (Nicholson and Setlow 1990). The media were then transferred to an incubator set at a temperature level of 37°C for five days. For separation of spores, the culture was centrifuged in 14000 rpm for 20 min (Sigma, Laborzentrifuge D- 31520 Germany).

Preparation of spore fractions and separation of crystalline CotA particles (CCP)

To prepare the spore fractions, two methods were used. A: preparation of crystalline CotA particles by heating, CCP (A). For this purpose the spore suspensions were heated at 100°C for 10 min in a boiling water bath. B: preparation of crystalline CotA particles by ultrasonic and autoclave, CCP (B). In this method, after a brief ultrasonic, spore solutions were autoclaved at 100°C /10 psi for 10 min. For separation of CCPs, samples were centrifuged in 14000 rpm for 20 min and the amount of proteins in mass and supernatant were assayed by Bradford method. Standard curve was obtained by bovine serum albumin (Bradford 1976).

Preparation of the support and enzyme immobilization

-Perlite activation

Twenty five gram of dry perlite that was separated with special mesh size was washed by stirring overnight in methanol to remove any organic contaminant and then was washed by distilled water. After that, 5 N NaOH was added to the cleansed perlite and the solution was heated for 30 min in a boiling water bath. Precipitated perlite was filtered and rinsed with water until NaOH removed (pH 7) (Torabi *et al*. 2007).

The same mass of the samples were added on activated perlite and dried at room temperature. Finally, perlite granules were washed to remove unattached particles. At the end of the immobilization procedure, neither enzyme activity nor protein was detected in the supernatant. It was confirmed that the samples were bounded to the support surface.

-Silanization of slides by Spin Coating

Removal of contaminants from glass slides was performed according to Labit *et al.* (2008) methods. For silanization, 1000 µl chloroform and 100 µl Vinyltrimethoxysilane were used. The organic solvent was removed by evaporation, and Vinyltrimethoxysilane was depositioned on the surface by Spin Coating in 2000 rpm for 2 min. The slides were placed overnight in a desiccator. After centrifugation in 14000 rpm for 20 min, mass of the samples was added on the surface and then was dried in a desiccator.

Study of enzymatic activities

-Laccase assay

Laccase activity was measured in 1ml reaction mixture containing 75 mM catechol as a substrate in 50 mM sodium phosphate buffer, pH 5 and 1 g of spore mass (4×10^{13} spores). One unit of laccase activity was defined as a change in A440 nm of 1 g in 1 min (Ruttimann *et al*. 1992).

-Catalase assay

To determine catalase activity, 500 µl of 3% hydrogen peroxide (H_2O_2) was added on spore biomass. After 10 min, 500 µl of 0.01% phenol red (pH 7.5) was added on the solution to detect the residual H_2O_2. One unit of catalase activity was defined as a change in A558 nm of 1 g spore biomass in 1 min.

- Phenol oxidase assay

The same mass of the samples was transferred to a 2 ml-microtube containing 200 µl of 1 M $NaHCO_3$ (pH 8.0) and Gibbs reagent (20 µl; 1 mg/ml in ethanol solution). The reaction mixture was shaken at 30°C for 30 min to allow the formation of a blue complex by the Gibbs reagent and hydroxyl groups of phenol (Yu *et al*. 2006). Percentage of phenol elimination was determined.

X-ray diffraction analysis (XRD)

X-ray diffraction analysis of the spore particles that dried at 30°C was performed with X-ray Diffractometer, D8ADVANCE (Bruker, Germany). X-rays were generated by a copper X-ray tube with Wavelength 1.5406 A° and Ni as a filter. Measurements were performed between 5° and 80° 2θ.

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectra of the samples were recorded on FT Infrared Spectrometer, JASCO, FT/IR-6300, Japan, in the range 4000–400 cm^{-1} by making the KBr thin pellet.

Results

Crystalline CotA particles

Table 1 shows the amount of protein and enzymatic activities of 1 g (4×10^{13}) spores and crystalline CotA particles (CCPs) after centrifuge in 14000 rpm for 20 min. As shown, the amount of protein in mass of CCP (A) that was prepared by heating was more than the other samples. Low amount of protein was observed in the supernatant of CCP (B).

Table 1. The amount of proteins and enzymatic activities of 1 g (4×10^{13}) spores and crystalline CotA particles (CCP)

Sample	Amount of protein (w/w)		Enzymatic activities		
	Mass	Supernatant	Laccase activity (Uni	Catalase activity (Uni	Phenol oxidase (Utiliz of phenol %)
***Bacillus* LPG-1 spores**	0.020	0	11.83	95	25%
CCP (A)[1]	0.046	0	14.16	112	45%
CCP (B)[2]	0.076	0.0017	10.5	100	30%
***Bacillus sphaericus* spores**	0.0239	0	1.2	64	0
Control [3]	0	0	0	0	0

1 preparation of crystalline CotA particles by heating

2 preparation of crystalline CotA particles by ultrasonic and autoclave.

3 Distillated water

Immobilization on perlite and silanized slide

The number of spores and amount of proteins after fixation are shown in table 2. During the immobilization process, some of spores and crystalline CotA particles that were not immobilized were lost by washing. About 32% and 20% of the bacterial spores were washed after immobilization on perlite and silanized slide, respectively.

Table 2. The number of spores and amount of proteins after immobilization

Sample	Number of spores		Amount of proteins (w/w)	
	Immobilized spores	Washed spores	Immobilized proteins	Washed proteins
Immobilization on perlite	2.72×10^{13}	1.28×10^{13}	CCP (A 0.027	0.012
			CCP(B 0.041	0.022
Immobilization on silanized slide	3.2×10^{13}	0.8×10^{13}	CCP (A): 0.037	0.008
			CCP(B): 0.056	0.014

Enzymatic activities

-Laccase, catalase and phenol oxidase activities in free samples

As shown in table1, laccase activity of B. LPG-1 spores was clearly more than *B. sphaericus* spores. Also, the highest laccase activity was observed in CCP (A). In previous study, we confirmed the structure of CCP by atomic force microscopy (AFM) and showed that these particles had high laccase activity (Subramanian, 2005). Catalase activity rate of *Bacillus* LPG-1 spores was higher than *Bacillus sphaericus* spores. Also, CCP (A) represented higher catalase activity than the other forms of spore. As shown in table 1, the maximum elimination of phenol due to phenol oxidase activity was observed in CCP (A) (45% in 30 min).

- Laccase, catalase and phenol oxidase activities in immobilized samples

The immobilized spores on silanized glass slide and perlite appeared to lose half of their laccase activity in comparison with free spores (Fig. 1). As shown in Fig. 2, catalase activity of immobilized spores was slightly lower than free spores. Since fewer spores were washed out during immobilization on silanized slide, enzymatic activity of silanized spores was higher than immobilized spores on perlite. As shown in Fig. 3, phenol oxidase activity of immobilized spores was also lower than free spores.

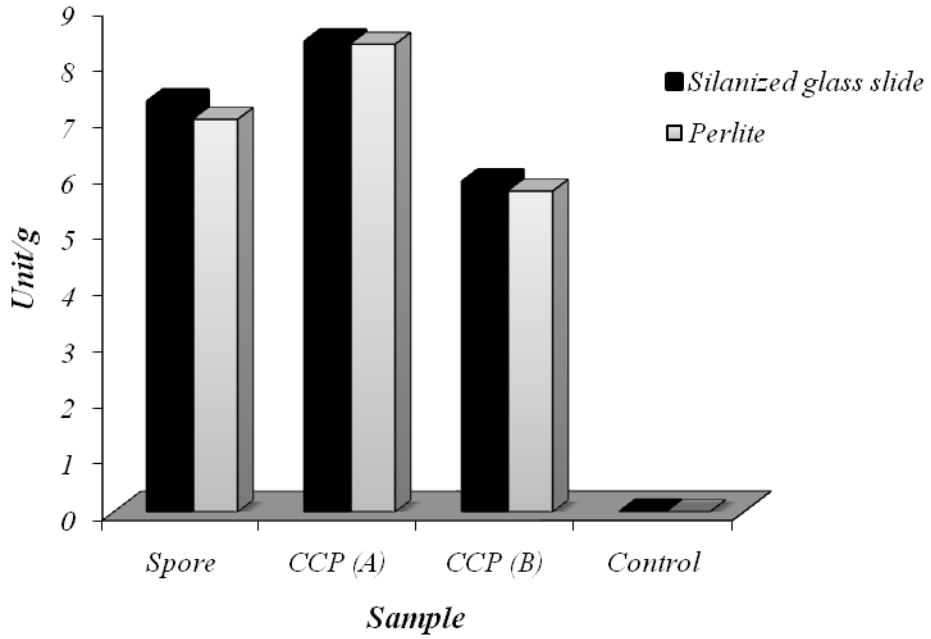

Fig. 1- Laccase activity in immobilized samples on glass slide and perlite. Spore: *Bacillus* LPG-1 spores, CCP: Crystalline CotA particles: A): was prepared by heating and (B): was prepared by ultrasonic and autoclave. Control: distillated water (The averages of three replications have been shown).

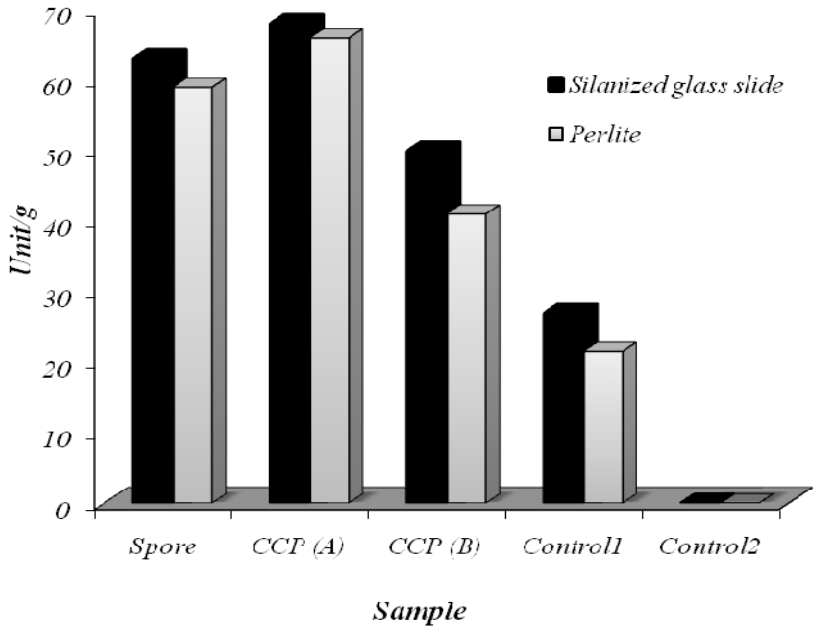

Fig. 2- Catalase activity in immobilized samples on glass slide and perlite. Spore: *Bacillus* LPG-1 spores, CCP: Crystalline CotA particles: A): was prepared by heating and (B): was prepared by ultrasonic and autoclave. Control: distillated water (The averages of three replications have been shown).

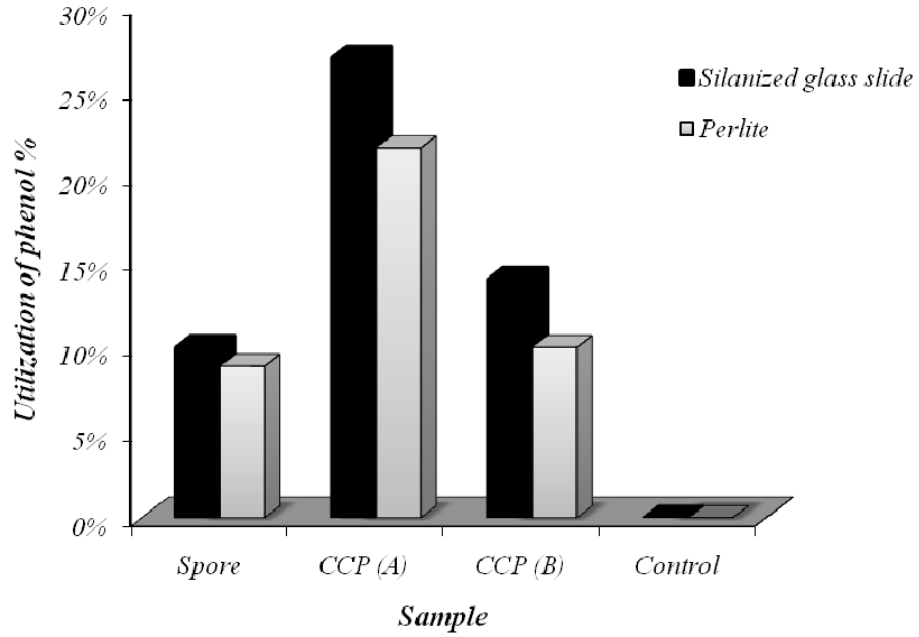

Fig. 3- Percentage of phenol removal in immobilized samples. Spore: *Bacillus* LPG-1 spores, CCP: Crystalline CotA particles: A): was prepared by heating and (B): was prepared by ultrasonic and autoclave. Control: distillated water. (The averages of three replications have been shown).

X-ray diffraction technique (XRD)

In this study, crystal structure of *Bacillus* LPG-1 spores was detected by XRD and also attachment of spores on silanized glass slide was confirmed by XRD. Fig. 4-A shows XRD image of free spores and B shows XRD image of immobilized spores on silanized glass slide. As shown, free and immobilized spores had a sharp peak about at 2θ= 15 ° and a broad peak about at 2θ=25°, respectively.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of spore and CCPs that are shown in Fig. 5 reflect the total biochemical composition of them. The region from 4000 to 3100 cm^{-1} consists of Amide A of proteins (N-H stretching). Amide I, amide II and amide III are appeared in 1700-1550 cm^{-1} and 1310-1250 cm^{-1}, respectively. Fatty acids are shown in 3100-2800cm^{-1} and phosphodiesters and carbohydrates are appeared between 1250- 800 cm^{-1} [13] Fig. 6 illustrates FTIR spectra of perlite and spore attachment to perlite. CCPs attachment to perlite is confirmed by the vibrational bands at 3441 and 1632 cm^{-1} in perlite which are shifted to the 3301 and 1655 cm^{-1} and also appearance of 2962, 1538, 1439 and 728 cm^{-1} bands.

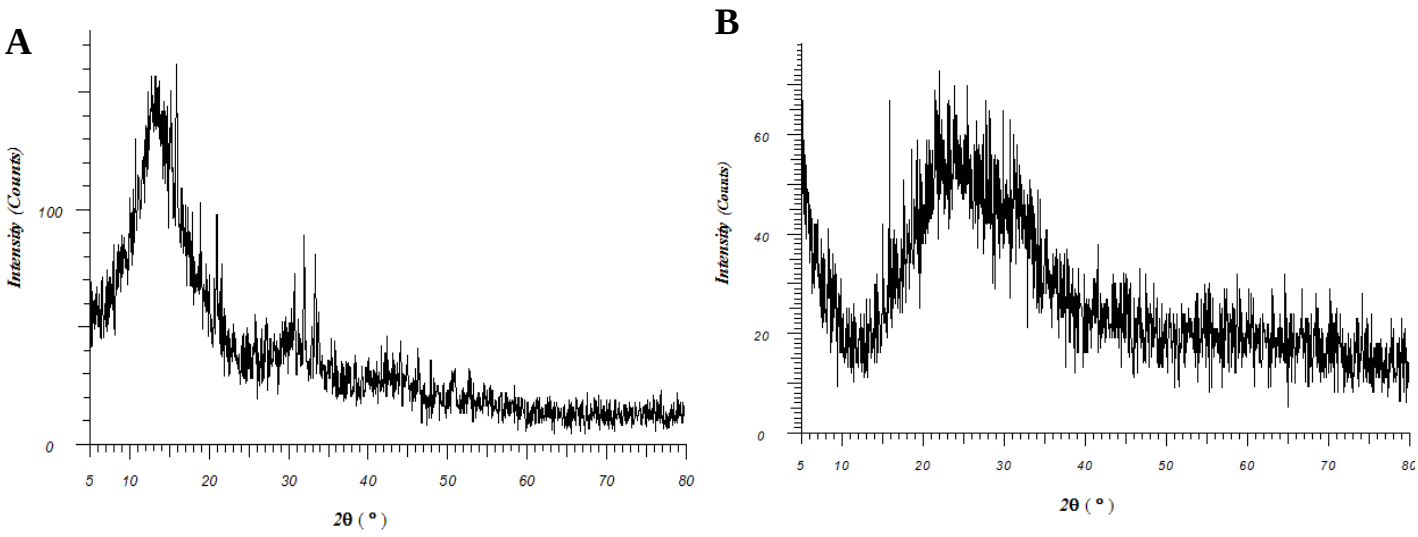

Fig. 4- XRD analysis of *Bacillus* LPG-1 spores. Free spores (A), Immobilized spores on silanized glass slide (B).

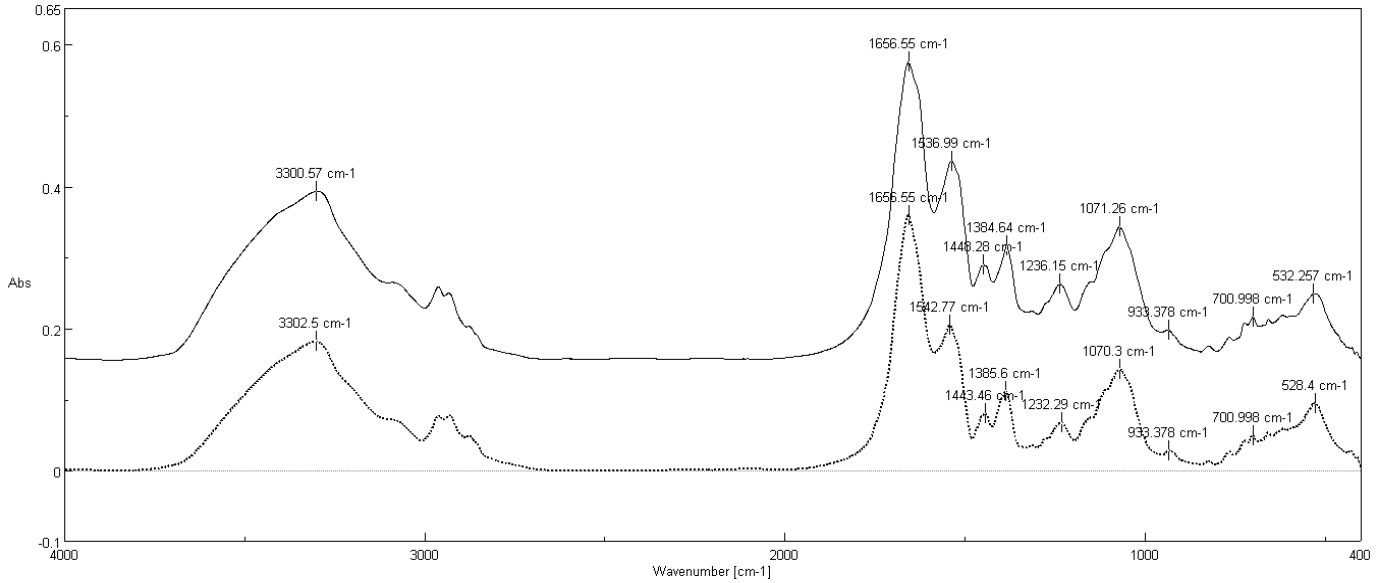

Fig. 5- FTIR spectra of spores of *Bacillus* LPG-1 (dashed line) and it's CCPs (solid line).

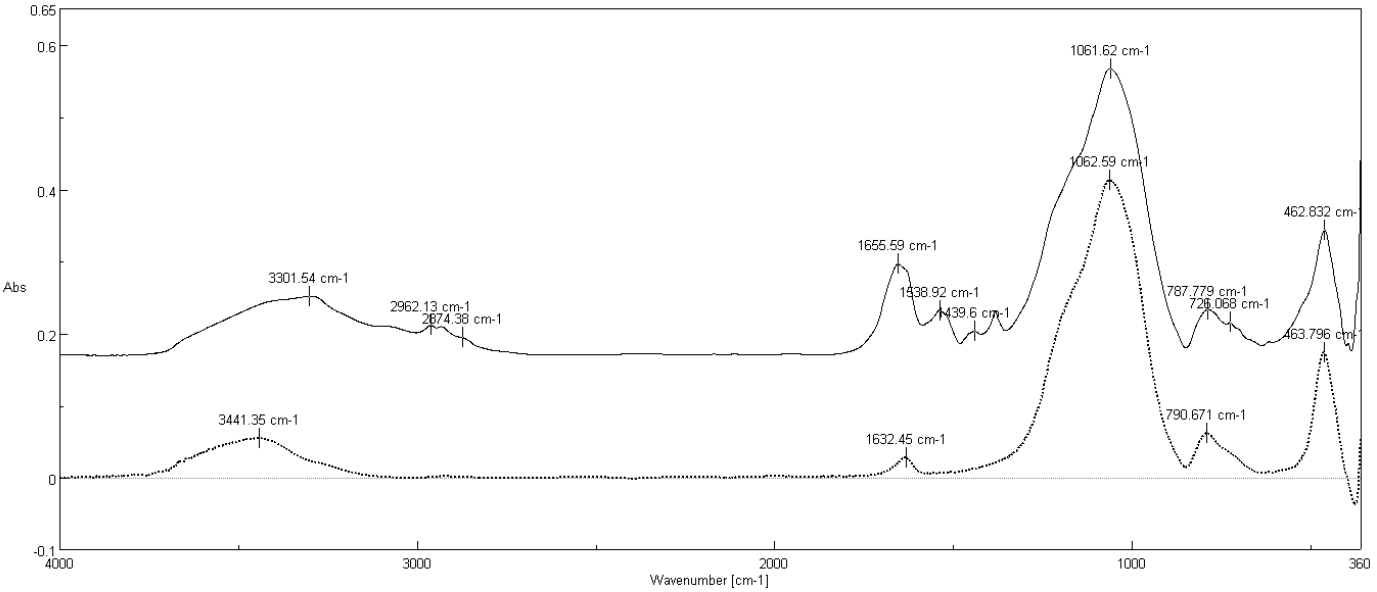

Fig. 6- FTIR spectra of perlite (dashed line) and immobilized CCPs on perlite (solid line).

Discussion

Several enzymatic activities have been observed in bacterial spores (Ruehle 1923). Coat proteins, especially CotA are responsible for their enzymatic activities (Enguita *et al*. 2003). In the present study, we observed that crystalline CotA particles of *Bacillus* LPG-1 spores can maintain their enzymatic activities at 100°C (Crystalline CotA particle A) and at 100°C/10 psi for 10 min (Crystalline CotA particle B). Enzymatic activities of CCPs were more than spores probably due to more availability of CotA particles on CCPs. Because of protein denaturation, it is obvious that the enzymatic activities of CCP (B) should be lower than CCP (A). Heat resistance and enzymatic activity of CotA was previously studied in other bacterial spores. Martins *et al.* (2002) showed that both spore-associated enzyme and purified protein of *Bacillus subtilis* endospore were heat stable laccase at 80°C for 4 h. Also, Reiss *et al.* (2011) reported heat stability of CotA protein of *Bacillus pumilus* up to 70°C for 20 min. Application of purified recombinant CotA to decolourize the azo dye Congo red in the treatment of textile industry wastewater was demonstrated by Zhang *et al.* (2012).

Since immobilization of enzymes makes them reusable, more stable against thermal denaturation, and also protects them from irreversible inactivation (Chong and Zhao 2004; Tischerw 1999; Torabi *et al*. 2007), choosing the appropriate methods for immobilization is very important to keep the catalyst active during the process. Perlite has many pours which keep the spores and increase their thermal tolerance. Silane plays a role as glue to keep the samples on the glass slides and prevent from washing.

Comparing with the free spores, it was shown that the procedure of immobilization lead to more reduction of enzyme activity. Enzyme immobilization via covalent binding will change enzyme conformation and reduce its activity (Chong and Zhao 2004; Torabi *et al*. 2007). Since CotA is located on the surface of spore, reduction in enzyme activity is due to covalent attachment of spores on perlite and silanized glass slide. Torabi *et al*. (2007) showed that covalent attachment of

cholesterol oxidase and horseradish peroxidase on perlite could decrease their activities.

Nowadays XRD is used to detect crystalline materials such as nanometals or proteins (Corte *et al*. 2011). In this study, X-ray diffraction was used for detection of immobilized spores on the silanized glass slides. The results of the X-ray diffraction are approximately similar to previous studies. In 1972, X-ray diffraction patterns of the *Bacillus subtilis* coat fractions were studied (Hiragi 1972). The coat fractions had two sharp peaks at about 2θ = 8.8° and 9.4°, and a broad peak at about 20°. In X-ray diffraction patterns of *Bacillus* LPG-1 (Fig. 4), free spores has a sharp peak at around 2θ= 15°. After immobilization on silanized slide the peak shifted and has a broad peak at around 2θ= 25°. *I*t is because of changes in crystalline structures of spores after covalent attachment on silanized slide. Immobilization can change the properties of the enzyme and the support that can easily be recognized by analyzing the XRD spectrum. Covalent attachment of the side chains of various amino acid groups in enzyme can cause significant changes in XRD peaks (Sanjay and Sugunan 2012).

FTIR as a simple and easy method has applied to analysis of bacterial spore structure (Subramanian *et al*., 2005). Attachment of spores to different solid surfaces such as bentonite has been determined by FTIR (Ammann and Brandl 2011). In our research attachment of CCPs to perlite was evidenced by changes in some bands of perlite (Fig. 6).

Last we propose that XRD and FTIR can be replaced with SEM (scanning electron microscopy), AFM (atomic force microscopy) and other expensive techniques to determine the effect of immobilization on enzyme activity and support properties.

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