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# Cloning and gene expression of cytochrome P450 gene from *Alcanivorax borkumensis* Bacterium

**Mehdi Hassanshahian [1, 2*], Mohammad Mehdi Yaghoobi[2]**

*1. Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran.*

*2. Research Dept of Biotechnology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, IRAN.*

## Abstract

*Alcanivorax borkumensis* is a marine bacterium that has ability to grow on limited substrates that mainly is alkanes. The ability to use wide range of hydrocarbons is advantage of this bacterium to other marine community bacteria. *A. borkumensis* have two genetic systems for alkane biodegradation. The First system is alkane hydroxylase (alk-B1 and alk-B2) and the second system is cytochrome P450. Until now there is not any report on cloning of cytochrome P450 gene of *A. borkumensis*. In this study cytochrome P450 gene from *A. borkumensis* was cloned. At first P450 gene was cloned in pBluescript plasmid by blunt cloning and insertion was confirmed by colony PCR. Then P450 gene was cloned in PET-26 expression vector and finally, IPTG induced gene expression in *E.coli* BL-21 bacterium. It was shown that longer induction time led to more expression level. The recombinant enzyme has difference absorbance in 450 nm that confirmed the activity of this recombinant enzyme.

**Key words:** Alkanes, Biocatalyst, Biodegradation, Cloning, Protein Expression

## Introduction

Petroleum hydrocarbons are widespread contaminants that enter to marine environment by crude oil transport, ships accidents and refinery process. Bioremediation is the best method for remediation of crude oil from marine environment. Some crude oil degrading bacteria were applied in bioremediation process. *Alcanivorax* is a crude oil degrading bacterium that is very important for oil biodegradation in marine environment (Hassanshahian et al., 2010; Emtiazi et al., 2005). *Alcanivorax borkumensis* first isolated in 1998 from sediments of North Sea, then this bacterium were isolated from different zones of the world such as Mediterranean Sea, Pacific Ocean and Sea of Japan and China. *A. borkumensis* is a marine bacterium that grows on limited substrates mainly alkanes (Yakimov et al., 1998; Yakimov et al., 2007).

*A. borkumensis* have two gene groups for degradation of alkanes includes: alkane hydroxylase system (alk-B2 and alk-B1) and cytochrome P450 system. Cytochrome P450 is an iron protein and this enzyme was found in plants, fungi, animals and bacteria. Cytochrome P450 has many applications such as

**Corresponding Author E-mail:** hasanshahi@gmail.com

metabolizing system for terpenes and alkanes and biodegradation of organic wastewater (Schneiker et al., 2006; Vanbeilen et al., 2006).

The application of *A. borkumensis* for biodegradation has some limitation such as slow growth and bacterium inability to metabolize the carbohydrate. One possible way to increase alkane degradation by this bacterium is the transfer the genes responsible for alkane degradation into fast growing bacteria (Hassanshahian et al., 2008; Hassanshahian el al., 2012). .

Cytochrome P450 enzymes are superfamily of hem enzymes and their sequences have been identified in variety of organisms, including mammals, organic plants, algae, fungi and bacteria. Vanbeilen et al (2006) by constructing a polycistronic vector transferred cyp153 into *P. putida* and recombinant strain can grow on alkanes (Vanbeilen el al., 2006). Kubota et al (2005) isolated cyt-P450 gene from different contaminated environments such as soil polluted with crude oil and groundwater (Kubota et al., 2005).

The purpose of this study is the transfer of cyt-P450 gene from *Alcanivorax* to *E. coli* to produce recombinant bacteria. This recombinant bacterium can be used to produce recombinant protein of cytochrome P450 as bio catalyst for hydroxylation of alkanes.

## Materials and Methods

### Bacterial, hosts and primers

The cyt-P450 gene (4211635) of *Alcanivorax borkumensis* strain SK2 was used for cloning. The host bacterium for cloning was *E. coli* DH5α and the *E. coli* BL21 were used as host for protein expression. In this study pBluescript plasmid was used as vector for cloning and pET-26 plasmid used as expression vector. The primer that used in this study was as follow: 201F: 5′CATATGTCAACGAGTTCAAGTAC3′ and 201 R: 5′ TTATTTTTTAGCCGTCAACTTAA 3′.

### Amplification of cyt-P450 gene

The cyt-P450 gene was amplified by PCR. PCR thermo cycling (Eppendorf AG 22331 Hamburg) protocol consisted of 30 cycles: denaturation of 1 min at 94°C, 1 min of annealing at 53 °C , and elongation of 1 min at 72°C followed by final extension step of 10 min at 72°C. Reactions were performed in a total volume of 50 µl containing 2 mM $MgCl_2$, 1X PCR reaction buffer (200 mM Tris; 500 mM KCl), 0.2 mM of each dNTP, 10 Pmol/µl of each primer and 1U of Taq DNA polymerase. PCR products (1413 bp) were visualized by gel electrophoresis using a horizontal 1 % agarose gel (Sigma, St. Louis, MO) with 1X TBE buffer. Gels were stained in a solution of ethidium bromide and visualized under a UVP UV transilluminator (UVP Inc., San Gabriel, CA). (Sambrook & Russel, 2001).

### Cloning of cyt-p450 into pBluescript plasmid

The pBluescript plasmid was extracted from *E. coli* DH5α by Metabion kit (Kit number: K12768) according to manufacture instructions. pBluescript plasmid linearized by EcoRV restriction enzyme. The cyt-P450 gene was inserting into pBluescript plasmid by ligation method. The ligation reaction carried out as follow: the total reaction was 25µl and contained 0.4µg of cyt-P450 gene with 1 unit of T4 DNA ligase enzyme (Qiagen) and 1X buffer (Qiagen) and 50 ng of plasmid that incubated overnight at 16 °C. The competence cells were constructed according to the protocols described by Sambrook and Russel, (2001). Recombinant plasmid was transformed into competence cells by heat and shock method. Blue/white screen carried out for selection of recombinant bacteria that 50 µl of transformed *E. coli* DH5α were cultured in LB media containing X-Gal, IPTG and ampicillin antibiotic (10 $mgml^{-1}$). The

plates were incubated for 24 hours in 37 °C. The white colonies were selected and for confirmation of recombinant bacteria, colony PCR was performed with specific primers of cyt-P450 gene.

**Expression of cyt-P450 gene in *E. coli* BL-21**

The *E. coli* BL-21 were used as host for expression of recombinant protein. The competence cells of *E. coli* BL-21 were prepared according to Sambrook and Russel, (2001) protocols. For transformation of *E. coli* BL-21, heat shock method was used. The cyt-P450 gene was inserted into pET-26 plasmid. The recombinant pET-26 plasmid was transformed into BL-21 host. The recombinant colonies of BL-21 were screened in media containing kanamycin antibiotic. For confirmation of recombinant colonies, PCR was carried out with specific primers of cyt-P450 gene. For induction of protein expression the IPTG (0.5-1 mM) were added and bacteria grew 4-5 hours. In one hour duration time 500 µl of sample were collected and suspended with SDS-PAGE buffer then froze. Vertical electrophoresis was performed for differentiation of proteins (Sambrook & Russel, 2001).

**Measuring the enzyme activity of recombinant cyt-P450 enzyme**

The recombinant E. coli BL-21 bacterium (containing pET-26 plasmid) was cultured in 250 ml of LB medium with kanamycin antibiotics at 37 °C on shaker (150 rpm). IPTG (1 mM) were added to culture medium when optical density of bacteria reach 0.3 at 600 nm and incubated at 37 °C for 8 hours. Also similar culture with the same condition was prepared except that IPTG not added and used as control. The bacterial suspensions were centrifuged at 5000 g for 20 min in the end of incubation period. The bacterial biomass was exposed to hexadecane for 4 hours to stimulate the enzyme activity of cyt-P450, and then the bacterial cells were broken with ultrasonic. The suspension were centrifuged and supernatants (stimulated and none stimulated) were used for enzyme activity assay (Nodate et al., 2006). The enzyme activity was measured by difference spectroscopy method. In this method the Carbon Monoxide (CO) gas bind to reduced enzyme and cause the difference absorbance in comparison to blank at 450 nm (Nodate et al., 2006).

**Results**

**Amplification of cyt-P450 gene**

Figure (1) shows the result of PCR amplification of cyt-P450 gene. As shown in this figure the expected band (1413 bp) successfully amplified.

**Cloning of cyt-P450 into pBluescript plasmid and transformation into *E. coli* DH5α**

White colonies with recombinant plasmid had higher molecular weight than blue colonies which confirmed the insertion of expected fragment into plasmid vector (Figure 2).

**Colony PCR in recombinant colonies**

To confirm, the insertion of cyt-P450 gene into recombinant colonies (white colonies) the colony PCR with specific primers of this gene was performed. The negative control of this experiment was blue colonies. As shown in figure (3), all white colonies have the cyt-P450 gene however this gene don't present into blue colonies that confirmed the successfully transformation of this gene into competence cells.

**Expression of cyt-P450 gene in E. coli BL-21 vector**

The expression of cyt-P450 protein was analyzed by protein electrophoresis and spectroscopy absorbance. As shown in figure (4) when the induction time with IPTG increased the expression of cyt-P450 protein show increment patterns (Fig 4). The absence of this protein band in unrecombinant plasmid confirmed that this protein only expressed in recombinant plasmid.

**Enzymatic activity assay of recombinant cytochrome P450**

The activity of cyt-P450 enzyme was determined measuring the difference in optical absorption between the enzyme reduction (reference) and a reduced enzyme that bind to the carbon monoxide complex. If the CO is connected to the reduced enzyme the catalytic cycles of enzyme are disrupted and the enzyme reaction that makes the difference absorption at 450 nm absorption is the highest. While the reductions form of the enzyme when it is not connected to the CO absorption does not show this feature. Thus, the free enzyme and the enzyme that bind to CO complex (reduced form) have different patterns. The result of enzyme assay was shown in figure (5), as shown in this figure the reduced form of cyt-P450 that bind to CO have the maximum absorbance at 450 nm while the reduced form that don't bind to CO hadn't absorbance in this area, thus recombinant enzyme has activity. The 420 nm peak related to attachment of enzyme and substrate.

**Discussion**

*A. borkumensis* is a marine bacterium that grows on alkanes and this matter cause the prevalence of this bacterium to other member of marine microbial community (Cappello et al., 2006; Humphreys et al., 2004). *A. borkumensis* have several systems for catabolism of hydrocarbons the first system is alkane hydroxylase and the second is cytochrome P450 system. This bacterium has three cytochrome P450 include cyt-P450a, cyt-P450b and cyt-P450c. these two systems have major role in biodegradation of hydrocarbons (Kubota et al., 2005).

Schneiker et al (2006) reported complete genome of *A. borkumensis.* They concluded that this bacterium has complex genetic system for response to oil pollution (Schneiker et al., 2006). There are two reports for cloning of hydrocarbon biodegradative gene in *A. borkumensis.* Smits et al (2002) cloned the alk-b1 gene of *A. borkumensis* strain AP1 in *E. coli.* They sequenced cloned gene and compared them with the alk-B genes from other bacteria and concluded that the alk-b1 gene of *A. borkumensis* is similar to alk-B gene of *P. oleovorans* GPO1 (Smits et al., 2006).

Hara et al (2004) cloned alk-b1 and alk-b2 gene of *A. borkumensis.* Their results show that the alk-b1 gene is responsible for biodegradation of hexadecane.

In this study firstly the cyt-P450 gene of *A. borkumensis* was amplified and transferred to pBluescript vector and recombinant vector was cloned and amplified in *E. coli* DH5α, Secondly, cyt-P450 gene was transferred to expression vector (PET-26) and cloned in E.coli BL-21 Finally, expression of this gene was induced by IPTG. Kim et al (2007) cloned *cyP52A2* gene of *Candida albicans* to *E. coli*. They expressed recombinant protein and used difference spectroscopy method to assay the activity of recombinant enzyme (Kim et al., 2007).

Kubota et al (2005) cloned cyt-P450 to construct a chimeric protein. They inserted this chimeric gene into pRED vector and expressed in into *E. coli* BL-21. Their results show that this chimeric protein could transform the alkanes to alkanol .The difference our study with research done with Kubota is they use

pRED vector for expression but we use pET-26 for expression. The pET-26 is better vector for low PCR product (1400 bp) then we used this vector. Nodate et al (2006) cloned cyt-P450 gene from Rhodococcus to pRED vector and expressed it in *E.coli*. They assay enzyme activity with GC-MS method by conversion of octane to octanol (Nodate et al., 2006). We used spectrophotometer absorbance of CO for assay of recombinant enzyme but the enzyme activity of our recombinant enzyme in comparison with other researcher was lower. It can be attributed to the following reasons:

1) In this study one gene from a marine bacterium cloned into non marine bacterium, it may be possible that the optimum condition for enzyme activity not present in the non marine bacterium.

2) Cloning in another host may alter protein folding of cyt-P450
3) It is possible that the bacterial cells haven't broken properly.

4) Codon optimization is an important challenge in this cloning and must be consider in future studies.

## Acknowledgment

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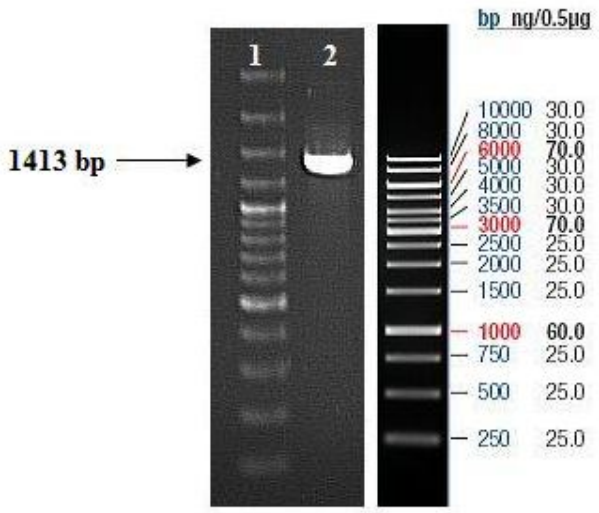


Figure 1. Gel electrophoresis of PCR product of cyt-P450 gene with specific primers lane (1) DNA size marker ladder (100bp) lane (2) PCR product

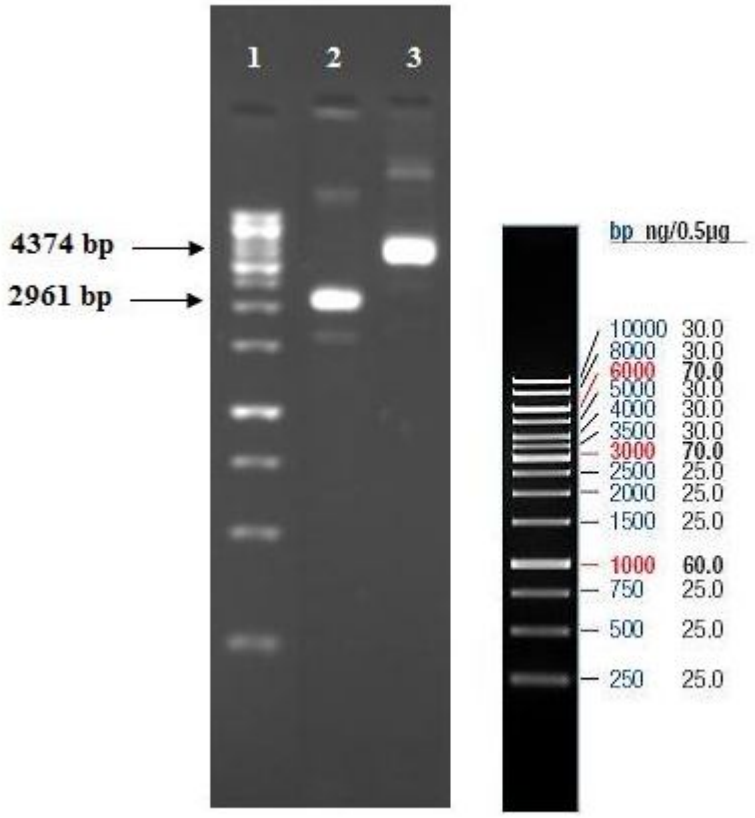


Figure 2. Gel electrophoresis of extracted recombinant plasmid. Lane (1): DNA size marker ladder (100 bp), lane (2): blue colony that contains only pBluescript plasmid and lane (3): white colony that contain recombinant pBluescript plasmid.

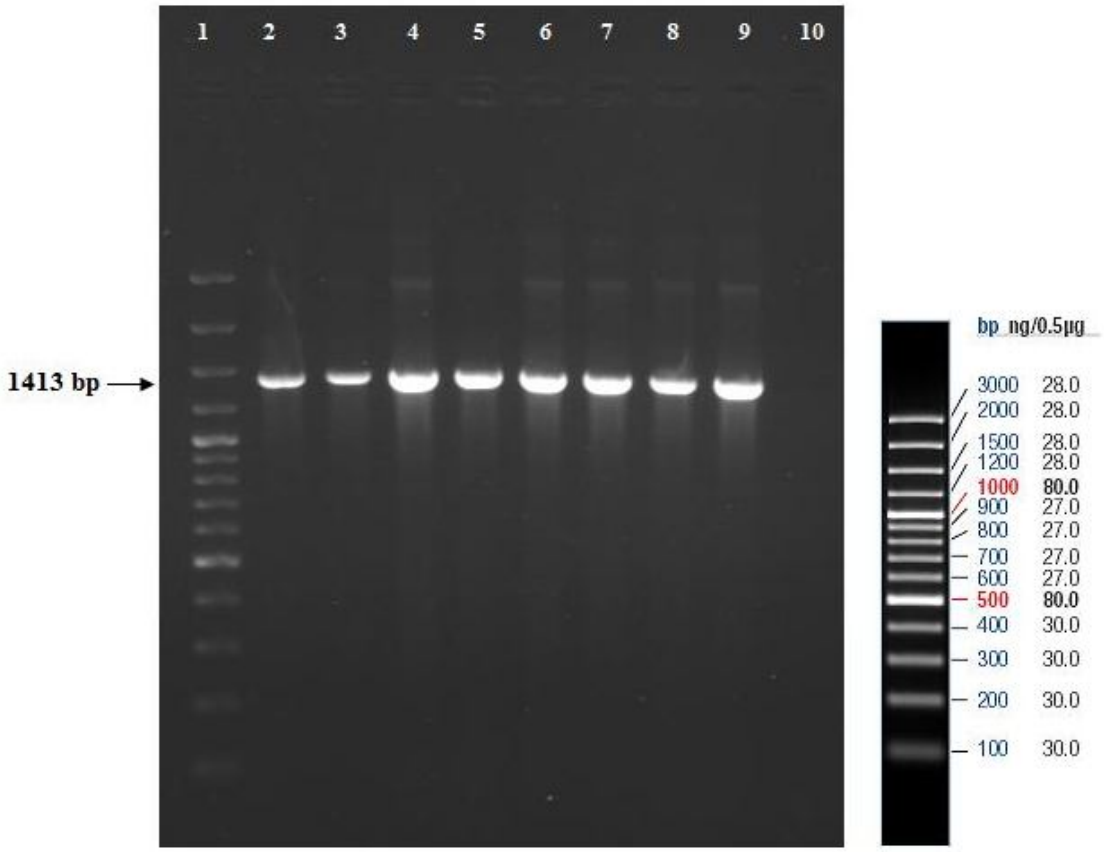


Figure 3. Confirmation of the cyt-P450 gene presence in recombinant colonies. Lane (1): DNA size marker ladder, lane (2-9) white colonies and lane (3): blue colony (negative control).

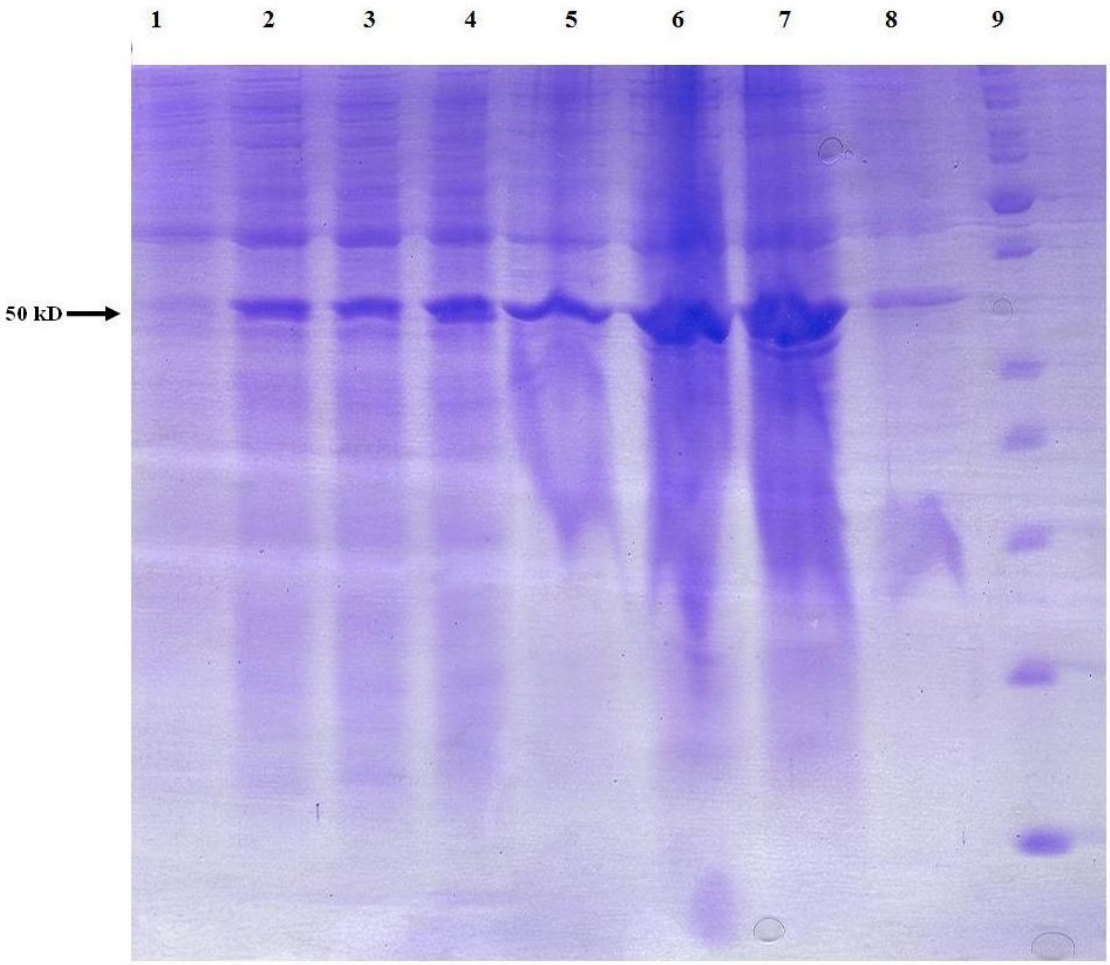


Figure 4. Protein electrophoresis of bacterial suspension contain recombinant pET-26 plasmid and not recombinant vector in SDS-PAGE gel. Lane (1-7) recombinant bacterium that exposed to IPTG induction at different times (time 0 lanes 1 to time 24 h lane 7), lane (8) not recombinant bacterium (as negative control) and lane (9): protein size marker ladder

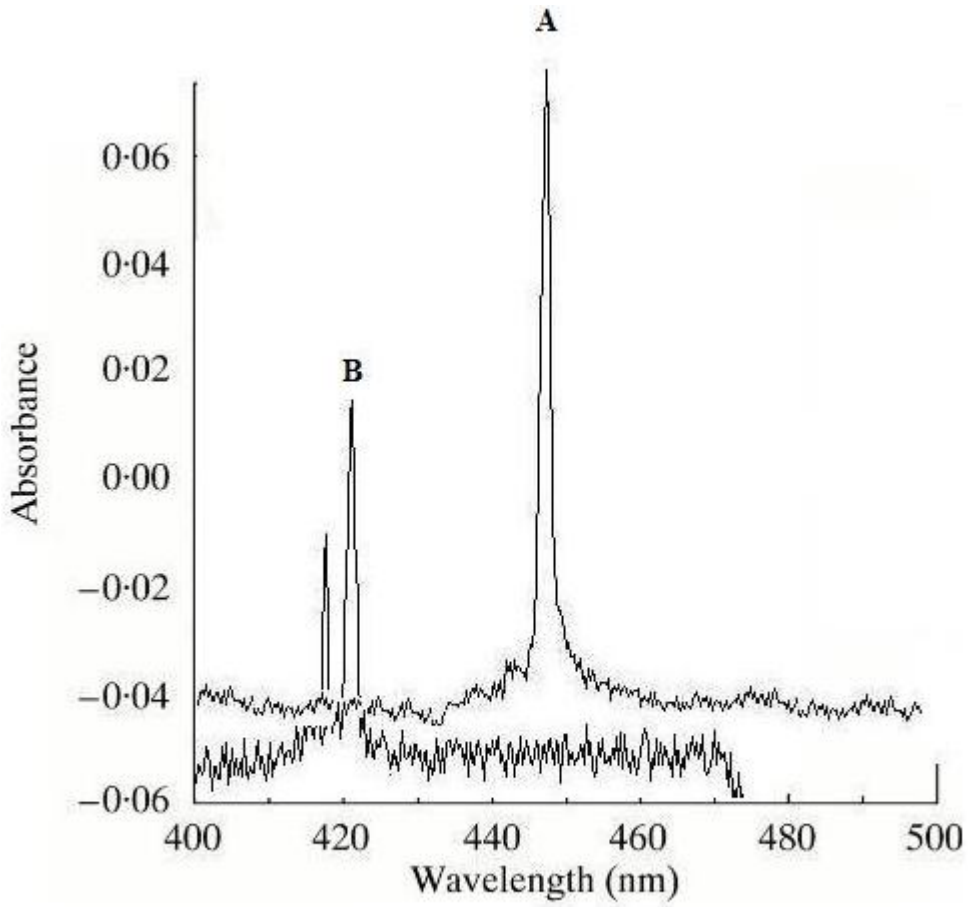


Figure 5. The difference between the optical absorption reductions of the enzyme is attached to the carbon monoxide complex (A) and reduced enzyme as reference (B).