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Biodegradation of polyaromatic hydrocarbons by recombinant bacteria containing Alasan gene

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Abstract: Alasan, the bioemulsifier of *Acinetobacter radioresistens* KA53, is a high molecular-mass complex of an alanine-containing polysaccharide and three proteins. This gene was isolated from the *A. radioresistens* using PCR and the amplified product (850 bp) was cloned using PGEMT cloning kit. DNA sequence was carried out for the amplification and sequence analysis showed that the PCR product was highly similar to the OMPA precursor protein which called *Alan*. Subcloning was carried out into pTRAC expression vector and the purified protein was used in mineralisation of the polyaromatic hydrocarbon. As well as the recombinant *E. coli* cells were cultured directly on MSM containing four polyaromatic hydrocarbon using real time method (phenol, naphthalene, phenantherene and antherathene). The obtained results showed that no difference between the purified protein and the using of the recombinant cells directly with the presence of the inducer like IPTG. Both of the purified protein and the recombinant cells showed high degradation rates for (anthrathene and phenantherene).

Keywords: Alasan gene; bacteria; biodegradation; PCR; polyaromatic; hydrocarbons.

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1 Introduction

Emulsifiers are surface-active agents that assist in the dispersion of one liquid to another, e.g., oil-in-water (o/w). Microorganisms synthesise a wide variety of high- and low molecular- mass bioemulsifiers (Rosenberg and Ron, 1997). The high-molecular-mass bioemulsifiers, referred to as bioemulsions (Ron and Rosenberg, 2001), are confirm polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers that stabilise oil-in-water emulsions. Bioemulsifiers are effective in lowering surface and interfacial tensions and high- molecular-weight polymers, referred to as bioemulsans, which bind tightly to surfaces (Rosenberg and Ron, 1997, 1999).

Although most researches on bioemulsans have focused on potential industrial and environmental applications (Klekner and Kosaric, 1993; Patel and Gopinathan, 1986; Robinson et al., 1996; Shepherd et al., 1995; Volkering et al., 1997), there is also a growing interest in the natural role of bioemulsans for the producing micro-organism. A large number of bacterial species from different genera produce extra-cellular bioemulsifiers. This property is particularly common amongst micro-organisms that degrade water- insoluble hydrophobic compounds, such as petroleum. Bioemulsifiers are used in microbial enhanced oil recovery and oil pollution remediation (Banata, 1995; Sar and Rosenberg, 1983). For better understanding how bioemulsans function in the growth and survival of micro-organisms, it is essential to elucidate their detailed chemical structures as well as the genes required for their biosynthesis. The majority of *Acinetobacter* strains, including both hospital and environmental isolates, produce

bioemulsans (Kaplan and Rosenberg, 1982). The best studied are the bioemulsans of *Acinetobacter calcoaceticus* RAG-1, *A. calcoaceticus* BD4, and *Acinetobacter radioresistens* KA53 (Navon-Venezia et al., 1995; Rosenberg et al., 1979; Belsky et al., 1979). A complex of an anionic heteropolysaccharide and protein is called RAG-1 emulsan (Zosim, 1989), its surface activity is largely due to the presence large amount of fatty acids, comprising 15% of the emulsan dry weight, which are attached to the polysaccharide backbone via O-ester and N-acyl linkages (Volkering et al., 1997; Navon-Venezia et al., 1998).

Alasan, the bioemulsifier of *Acinetobacter radioresistens* KA53, is a high molecular-mass complex of an alanine-containing polysaccharide and three proteins (Toren et al., 2001). The protein fraction is essential for emulsifying activity and maintaining the structure of the complex (Rosenberg et al., 1979). Separation and purification of the three Alasan proteins demonstrated that one of the proteins, with an apparent molecular mass of 45 kDa, was the surface active component of the complex (Toren, 2002). The 45 kDa protein had a higher specific emulsifying activity than the Alasan complex. The gene coding for the 45 kDa protein was cloned, sequenced and expressed in *Escherichia coli* (Barkay et al., 1999). The recombinant protein AlnA (35.77 kDa) without the leader sequence) had an amino acid sequence homologous to the *E. coli* outer membrane protein A (OmpA) and contained the emulsifying activity of the active 45 kDa glycoprotein. In addition to their emulsifying activities, Alasan, the 45 kDa protein and recombinant AlnA were highly effective in solubilising polyaromatic hydrocarbons (PAHs) (Toren et al., 2002; García-Junco et al., 2001). Furthermore, Alasan stimulated the growth of *Sphingomonas paucimobilis* on phenanthrene and fluoranthene (Carolan et al., 2005).

Fluorescence is a standard analytical technique that can be used to measure the concentration of various analyses in many different matrices. For PAHs, only UV light is required to excite the emission of visible light (Resina-Pelfort et al., 2003). When UV light is passed through a sample, the sample emits light (fluoresces) proportional to the concentration of the fluorescent molecule (in this case, PAHs) in the sample. UVS is based on the measurement of fluorescence observed following UV excitation of organic solvent presented in sample (Sambroo et al., 1989). This method, however, makes it possible to improve detection limits by several orders of magnitude to the range of 1–5 ppm total in culture media containing PAH. Because many different PAHs can fluoresce, this method is able to quantify individual PAHs, but can serve to screen for bulk PAH levels in the culture media (Andrew and McCracken, 2003).

In this paper, cloning, sequencing and over expression of the gene (*AlnA*) were performed. In addition, examination of recombinant strain to synthesis emulsifier protein was achieved.

2 Experimental

2.1 Amplification and cloning the AlnA gene

All PCR amplifications were performed using a 9700 PCR thermal cycler (Berkenelemer, USA). Plymerase Chain Reaction mixtures were prepared with 5 µl of Taq buffer 10X 2.5 mM of MgCL2, 200 µmol of each deoxynucleoside triphosphate,

20 Pmol each primer, [forward: ′5-TGG CGA CCT GAC TGA TAG CGT AGA ACT-′3 and reverse primer: 5 –GAG CAG TCA AAC GAG ATG GAT CAA TAT-′3.] 5 µg of bovine serum albumin, 1% of formamide and 2.5 U Taq polymerase (Promega, Germany) and sterile filtered milliQ water to a final volume of 50 µl. The PCR program was as follows: denaturing step of 94°C for 3 min, followed by 35 cycles for 1 min at 94°C, annealing for 1 min at 60°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. The amplification products were routinely analysed by electrophoresis in 1.4% agarose gels in 1X Tris-borate-EDTA buffer (Bekerman et al., 2005). The PCR product was purified using gel extraction kit (Qiagene, Germany), and then the purified DNA was subjected transformation into *E. coli* DH5α using PGEM-T cloning kit (Promga) according to manufacture instruction. Subcloning was carried out for that gene into prokaryotic expression vector pTrcHis and in vitro transcription was carried out for the recombinant bacterial cells.

2.2 DNA sequence alignments and phylogenetic analysis

The PCR product was subjected to sequencing using automated sequencer 310 (ABI). Pairwise and multiple DNA sequence alignment were carried out using ClustalW (1.82) (http://www2.ebi.ac.uk/clustalw; Thompson et al., 1994). Bootstrap neighbour-joining tree was generated using MEGA 3 (Kumar et al., 2004) from CLUSTALW alignments.

2.3 Mineralisation of PAHs using the purified protein of the AlnA gene

About 280 µl of MSM media containing the desired polyaromatic hydrocarbon a combined with 20 µl of the purified protein. Samples from the culture were taken each one hour and the polyaromatic degradation was then tested using Flourosckan apparatus at excitation (490) and emission (960) (Ascent, Labsystem, Finland).

2.4 Degradation rate of E. coli cells contains the AlnA gene in prokaryotic expression vector pTRAC

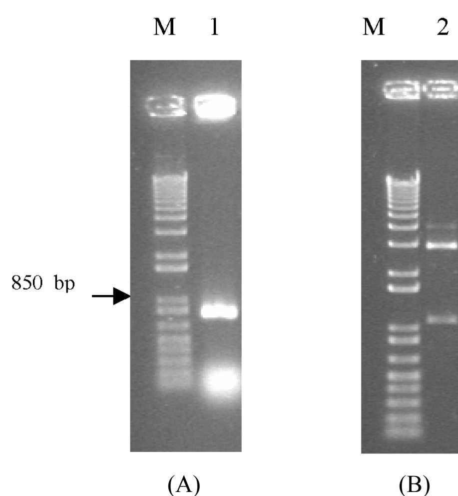
The degradation rate of the recombinant bacteria was examined on line using the Real time method with slight modifications (Winge et al., 1993). Solvent extraction requires additional time for sample preparation, so although fluorescence is said to be a real-time measurement, the total time for analysis may be 5 min hence the description of ‘real time’ is a fact. Results of UVS can be downloaded to Excel or GMS software. The PAH(s) were added to the MSM culture media (Mineral Salt Medium) as a carbon source separate or in combination. 270 µl from MSM containing the desired hydrocarbon (naphthalene, anthrathene, phenantherene and phenol) was loaded into 96 well ELISA plate (Coster, Germany), and then bacterial inoculums was added to each well (30 µl from fresh culture). The plate was incubated at the optimum temperature overnight in the Fluorosckan. The degradation values were monitored directly each one hour on the apparatus and the data was recorded automatically as Excel sheet.

3 Results

3.1 Amplification of the AlnA gene by PCR and cloning into PGMT vector

The AlnA was amplified using specific primers and approximately 850 bp were obtained as shown in Figure 1(A). The band was purified from the agrose gel using gel purification kit and cloned into PGEMT vector, data presented in Figure 1(B).

Figure 1 Agarose gel: Lanes M: 1 kb plus DNA ladder marker, Lanes 1 and 2 amplified Alasen fragment (A) and digested plasmid with *XbaI* and *HindIII* (B), respectively



3.2 Sequence and sequence analysis of Alasen clone

The amplified PCR product was subjected to sequencing using automated DNA sequencer and the data used in sequence analysis using DNA BLAST. The data revealed that the obtained sequence very closed to OmpA-like protein precursor, gene and submitted to gene bank under the accession No. (DQ435685). Further analysis was carried out using ClustalW and MEGA 3.1 programs to examine the homology between *AlnA* and the other sequences as show in Figure 2(A) and (B). The data revealed that *AlnA* is very close to AY033946 gene (*Acinetobacter junii* HMP-like outer membrane protein gene), which proves the obtained gene belong this family of the bioemulsifers and may be it has the vital component to be effective as a surfactants material.

Figure 2(A) Phylogenetic tree showing evolutionary relationship between the bioemulsifer *AlnA*

Figure 2(B) Comparative amino acid sequences analysis of cultivated *AlAn* clones with other Alasan ones using ClustalW. Names refer to the accession number of the nucleotide; identical amino acids are designated by (*)

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DQ435685      ----------------PVCWCSLGVDLLHGR-FEAEYSQIKGDTS---VQATNIRRK—Y  38
AY033946      GGNDGDLTDSVELDDDLFVGAALGVELTPWLGFEAEYSQIKGDTS---VAGNEYKGENIA 98
AF132598      NGNDGELTSSPELQDDLFVGAAIGVELTPWLGFEAEYSQVKGDVDGA-AEGAEYKGQNIA 119
AY626903      GGDKGELTAGPELQDDLFVGAALGVELTPWLGFEAEYNQVKGDLDGTGVAGSEYKQTQIN 72
                               .      ::**:*     *****.*:***  .    . . : :

DQ435685      RWLYVTSDLFTKNYDSKIKPYALLGAGHYKYEFDTDAGRAGRGLDEEGTLGNAGLGAFWR 98
AY033946      GNFYVTSDLFTKNYDSKIKPYALLGAGHYKYEFDTDAGRAGRGLDEEGTLGNAGLGAFWR 158
AF132598      GNFYATSDVFTGNYDSKVKPYMLLGAGHYKYEFEG-VPRGTRGNEEEGTLGNAGVGAFWH 178
AY626903      GNFYATSDLITKNYDSKIKPYVLLGAGHYKYKLDDMSYHN----NEEGTLGNAGVGAFWR 128
                :*.***::* *****:*** *********:::     :      :*********:****:

DQ435685      INDALSLRTEARGTYHFDEQFWNYTALAGLNVVLGGHLKPAAPVVEVVPVEPTP--IVEP 156
AY033946      INDALSLRTEARGTYHFDEQFWNYTALAGLNVVLGGHLKPAAPVVEVVPVEPTP—IVEP  216
AF132598      INDALALRTEARGTYHFDEKFWNYTALAGLNVVLGGRLKPAAPVVEVAPVEPVT--PVAP 236
AY626903      LNDALSLRTEARGTYNFDEKFWNYTALAGLNVVLGGHLKPAAPVVEVAPVVPVAPTPVEP 188
             :****:*********:***:*****************:**********.** *..    * *

DQ435685      QPQELTEDLNM-LRVFFDTNKSNIKDQYKPEIAKVAEKLVEYPNATARIDGHTDNTGPRA 215
AY033946      QPQELTEDLNMELRVFFDTNKSNIKDQYKPEIAKVAEKLVEYPNATARIDGHTDNTGPRA 276
AF132598      PPQELTEDLNMELRVFFDTNKSNIKDQYKPEIAKVAEKLVEYPNATARIEGHTDNTGPRA 296
AY626903      APQVLTEDLNMELRVFFDTNKSNIKDQYKPEVAKVADALNTFPNATARIEGHTDNTGPRK 248
               ** ******* *******************:****: *   :*******:*********

DQ435685      LNERLSLARANSVKSSLVNEYNIDPSRLTAQGFAWDQPIADNSTKEGRAMNRRVFATITG 275
AY033946      LNERLSLARANSVKSSLVNEYNIDPSRLTAQGFAWDQPIADNSTKEGRAMNRRVFATITG 336
AF132598      LNERLSLARANSVKSSLVNEYNVDASRLSTQGFAWDQPIADNNTKEGRAMNRRVFATITG 356
AY626903      LNERLSLARANSVKSALVSEYNIDASRLSTQGFAWD-----------------------  284
              ***************:**.***:*.***::******

DQ435685      INDALSLRTEARGTYHFDEQFWNYTALAGLNVVLGGHLKPAAPVVEVVPVEPTP--IVEP 156
AY033946      INDALSLRTEARGTYHFDEQFWNYTALAGLNVVLGGHLKPAAPVVEVVPVEPTP—IVEP  216
AF132598      INDALALRTEARGTYHFDEKFWNYTALAGLNVVLGGRLKPAAPVVEVAPVEPVT--PVAP 236
AY626903      LNDALSLRTEARGTYNFDEKFWNYTALAGLNVVLGGHLKPAAPVVEVAPVVPVAPTPVEP 188
              :****:*********:***:*****************:**********.** *..    * *

DQ435685      QPQELTEDLNM-LRVFFDTNKSNIKDQYKPEIAKVAEKLVEYPNATARIDGHTDNTGPRA 215
AY033946      QPQELTEDLNMELRVFFDTNKSNIKDQYKPEIAKVAEKLVEYPNATARIDGHTDNTGPRA 276
AF132598      PPQELTEDLNMELRVFFDTNKSNIKDQYKPEIAKVAEKLVEYPNATARIEGHTDNTGPRA 296
AY626903      APQVLTEDLNMELRVFFDTNKSNIKDQYKPEVAKVADALNTFPNATARIEGHTDNTGPRK 248
               ** ******* *******************:****: *   :*******:*********

DQ435685      LNERLSLARANSVKSSLVNEYNIDPSRLTAQGFAWDQPIADNSTKEGRAMNRRVFATITG 275
AY033946      LNERLSLARANSVKSSLVNEYNIDPSRLTAQGFAWDQPIADNSTKEGRAMNRRVFATITG 336
AF132598      LNERLSLARANSVKSSLVNEYNVDASRLSTQGFAWDQPIADNNTKEGRAMNRRVFATITG 356
AY626903      LNERLSLARANSVKSALVSEYNIDASRLSTQGFAWD--------------------   -284
                      ***************:**.***:*.***::******
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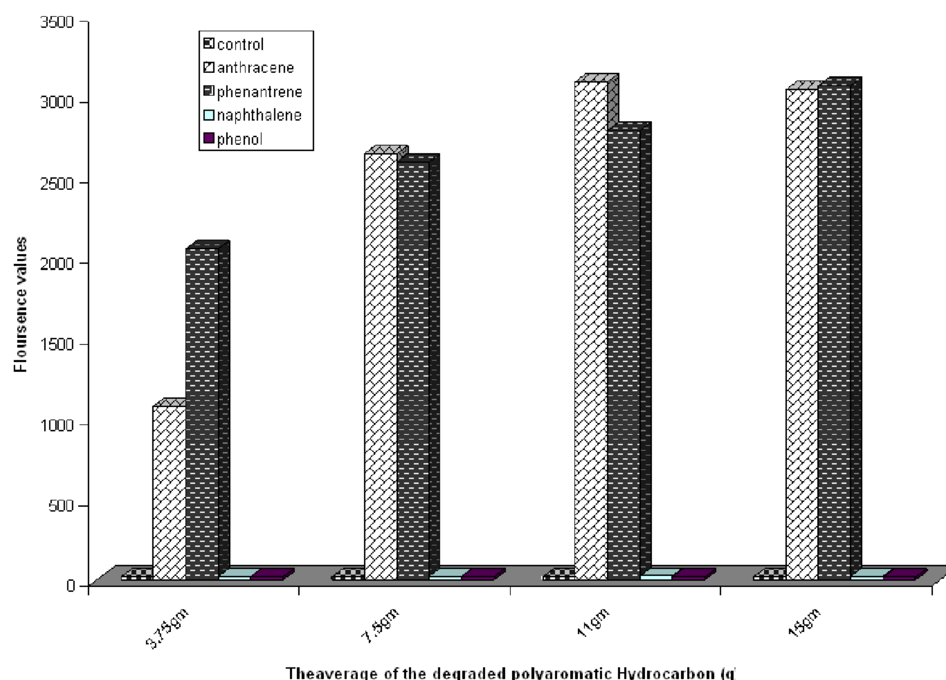
DNA sequence and the other emulsifier sequences, AY626903: HMP-like outer membrane protein gene, AF132598: ompA gene, AY033946:OmpA-like protein precursor, gene. The Neighbour-Joining method was used to construct the tree. The numbers on the branches represent bootsrap support for 1,000 replicates. Names refer to the accession number of the nucleotide.

3.3 Polyaromatic hydrocarbon degradation using AlaN pTRAC clones

After cloning of the Alasen gene into the prokaryotic expression vector pTRAC into the *E. coli* cells and screening was confirmed using PCR. The resultant clones were subjected

to a high concentration of four polyaromatic hydrocarbons as presented in Figure 3. The data obtained by on line method showed that the pTRAC clone able to degrade only antherethene and phenantherene but unable to degrade phenol and naphthalene. However, the resultant data prove that Alasan may be target specific in mineralisation of some polyaromatic hydrocarbons but has no effect on the others.

Figure 3 The degradation of the four polyaroamtic hydrocarbons using the transformed *E. coli* containing AlaN gene in pTRAC plasmid (see online version for colours)



4 Discussion

Isolation of the Alasan gene was carried out using specific primers to isolate approximately 850 bp from *Acinetobacter radioresistens* KA53 genomic DNA. The PCR product was then cloned using PGEMT Kit (Promega) and the clone was subjected to DNA sequence using automated sequencer (ABI, 310, Labbiosystem). Sequence alignment was carried out using BLASTn and the data showed that the AlnA is much closed to OmpA-like protein precursor which isolated also from *Acinetobacter radioresistens*. The isolated gene was shown that it has four open reading frames and it is very close to OmpA- like protein gene (AYO33946) as shown in Figure B2. The AlnB protein of the bioemulsan Alasan is a peroxiredoxin. AlnA has previously been shown to be an OmpA-like protein precursor that is largely responsible for the emulsifying activity of Alasan. To further elucidate the nature of Alasan, the gene coding for AlnA was cloned and over expressed in *E. coli*. Recombinant and the recombinant strain were then used to degrade the polyaromatic hydrocarbon as in mineral salt media (Lahlou et al., 1999).

UV fluorescence (UVS) spectrophotometer is a screening technique that can be used in the ecological risk assessment to rapidly estimate total PAHs levels in sediments and also in bacterial culture (Andrew and McCracken, 2003). It provides semi-quantitative data at less cost, which helps limit sampling and lab analysis to the area of concern, fill in data gaps, and ensures that expensive, certified analyses have the greatest possible impact. Traditional site characterisation is an expensive and time-consuming process. It will be considered as on line procedure of field sampling and laboratory analysis cycles, with subsequent sampling and analysis guided by previously described by Desai and Banat (1997).

In this paper, based on UV spectrophotometric determinations (Navon-Venezia et al., 1995; Rosenberg et al., 1979), we present data indicating that the bioemulsifier Alasan increases the apparent solubility of some PAHs, that this activity is likely due to a reversible binding of these compounds, and that its enhances the biodegradation of PAHs. On the other hands there are a multitude of reports on increased apparent solubility and biodegradation of the polyaromatic hydrocarbons (anthrathene and phenantherene) using Alasan. Results presented here in Figures 2 and 3 indicate that Alasan, increases apparent solubilities of PAHs and that the efficiency of this solubilisation is similar to those reported for synthetic surfactants (Doong and Lei, 2003). So, these biosurfactans might be used in PAH degradation if it is injected to the contaminated soils or waste water with antherathene and phenantherene. These results consistent with (Doong and Lei, 2003) state that the solubilisation and mineralisation of PAHs increased in a soil system amended with different surfactants (Doong and Lei, 2003).

5 Conclusions

We can conclude that, *Alan* has potential rates in degradation of the polyaromatic hydrocarbons especially antherethene and naphthalene is so high and willing. These data was confirmed by semi-industerial scale after induction of the gene and increasing its expression rate in the culture filtrate. In addition, *Alan* is able to degrade a certain types of PAHs and it is recommended to use in applications in the bioremediation of contaminated sites by accelerating the biodegradation rates of hydrophobic pollutants. Moreover, Alasan, being a high-molecular-weight biopolymer, can serve as a useful model system for the study of the way by which polymeric biosurfactants enhance the solubilisation of solid compounds with low aqueous solubility.

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