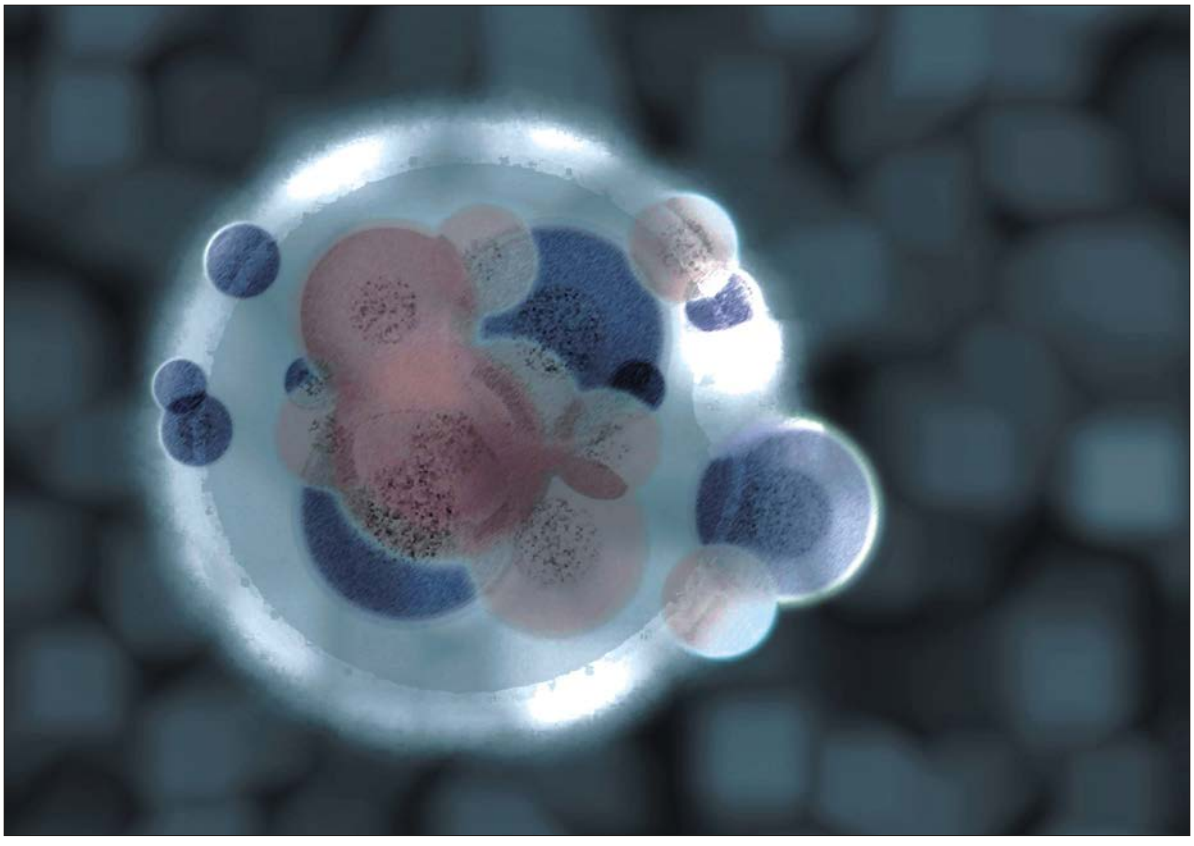

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The Effects of Various Immobilization Matrices on Biosurfactant Production using Hydrocarbon (HC)-Degrading Marine Bacteria via the Entrapment Technique

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Abstract: Bioavailability and environmental stress are problems affecting Poly Aromatic Hydrocarbons (PAH) biodegradation. This study aims to overcome the effect of PAH bioavailability via biosurfactant production and the effect of environmental stresses via using different immobilization matrices. Three different PAH bacterial degraders (A2, P5 and N7) were immobilized in different immobilization matrices. The immobilization matrices used in this investigation were: ca-alginate, agar-agar and agarose. RAPD-PCR, plasmid profile and 16S rRNA sequencing methods were used to identify and group the bacterial isolates. The production of biosurfactant was detected using the methylene blue analysis procedure. The results indicated that P5 and N7 isolates preferred the alginate matrix compared to the agar and agarose matrices, where biosurfactant production was 136 and 165.5 mg L^{-1} for both isolates, respectively. However, the A2 isolate produced a higher biosurfactant concentration (132.4 mg L^{-1}) when grown on agarose. The preferred matrices for the three isolates were different in the presence of hydrocarbons. The A2 and P5 isolates preferred agar as the best matrix for biosurfactant production. On the other hand, the free cells of the N7 isolate produced the highest concentration of biosurfactant compared to immobilized cells. The overall results of this study showed that the type of preferred immobilization matrix depends on the used carbon source; where, in general, calcium alginate was preferred at the presence of glucose while agar was preferred at the presence of hydrocarbons.

Key words: Cells immobilization, biosurfactant production, biodegradation, hydrocarbon, 16S rRNA gene

INTRODUCTION

Poly Aromatic Hydrocarbons (PAHs) are a group of large and diverse organic compounds composed of two or more fused aromatic rings in linear, angular and cluster arrangements (Mrozik *et al*., 2003). The main sources of PAHs are industrial production, vehicle emissions, power plants, transportation, refuse burning and spillage of petroleum products (Makkar and Rockne, 2003). Different State and Central Pollution Control Boards have identified PAHs as hazardous chemicals because of their toxic and carcinogenic effects on the living body (Ruma *et al*., 2007). They are detected in surface and ground water (Holman *et al*., 1999) air, (Lim *et al*., 1999), soil and sediment (Rockne *et al*., 2002).

Various chemical (e.g., chemical oxidation) and physical (e.g., thermal desorption and solvent extraction) applications have been utilized for the treatment of environments contaminated by petroleum hydrocarbons (Piskonen and Itavaara, 2004). However, these treatments are expensive and often only change the contaminants to other forms (Ward *et al*., 2003). Alternatively, microbiological biodegradation can be an effective and inexpensive approach to remediating environments containing PAHs and other hydrocarbon compounds (Phillips *et al*., 2000). Moreover, the use of microbes can convert toxic or persistent organic molecules into harmless products such as carbon dioxide and water as end products (Ruma *et al*., 2007).

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The rate of *in situ* microbial metabolism of PAHs can be limited by the low solubility of these compounds resulting in low bioavailability to the microbial degraders (Makkar and Rockne, 2003). Surface-active agents (surfactants) are used as mobilizing agents to improve the solubility of PAHs (Ron and Rosenberg, 2002). Biological reactions occur in or at the interface of the aqueous phase and surfactants have the ability to desorb and disperse poorly soluble compounds in small, high-surface-area micelles within the water phase. Consequently, surfactants can enhance PAH biodegradation by improving the accessibility of these compounds to microbial attack (Timmis and Pieper, 1999).

The use of free bacterial cells for bioremediation of contaminated sites might fail since the inoculants must be able to overcome biotic and abiotic stresses in the contaminated environment and thus might cause other problems such as secondary pollutants (Gentili *et al*., 2006). Immobilized microorganisms have been shown to be effective and have been receiving increasing attention (Tao *et al*., 2010). Several advantages of using immobilized microorganisms over free cells, including being able to protect microbial degraders from the toxic effects of hazardous compounds and improving their survival and metabolic activity during bioremediation (Moslemy *et al*., 2002). Calcium-alginate is one of the most commonly used immobilization methods because the procedure is simple, relatively mild and does not have any toxic effects on the cells (Sergio and Bustos 2009; Tao *et al*., 2009).

The degradation of naphthalene using immobilized *Pseudomonas* sp. in several different matrices, including K-carrageenan, alginate, agar, polyacrylamide hydrazide and polyurethane foam, has been previously investigated (Manohar *et al*., 2001; Seoud and Maachi, 2003). However, it was found that the alginate method has several advantages over the other immobilization methods, including being mild, fast, simple and cheap immobilization method (Feijoo-Siota *et al*., 2008). In addition, the stability of alginate spheres and the prevention of their breakdown are important factors for the proper long-term functioning of immobilized beads. However, viscosity is considered as the main limitation in the use of alginate beads (Darrabie *et al*., 2006). Therefore, the aims of this study were to isolate and identify indigenous biosurfactant-producing bacteria that are capable of degrading PAHs and to investigate the effect of the immobilization method on the rate of *in situ* PAHs biodegradation.

MATERIALS AND METHODS

Bacterial isolates: Three bacterial isolates were previously isolated from three PAH polluted sites in the Mediterranean Sea, Alexandria, Egypt, in January 2011. One isolate was able to degrade phenantherene as a sole carbon source and with an energy source up to 150 mg L^{-1} and labeled as (P5). The other two isolates were able to degrade naphthalene and anthracene as a sole carbon source up to 450 and 110 mg L^{-1} and were labeled as N7 and A2, respectively.

Bacterial cell immobilization

Inoculum preparation: The three bacterial isolates P5, N7 and A2 were cultivated at 30°C on a rotary shaker for 24 h in nutrient broth medium containing 0.15% (w/v) beef extract, 0.15% (w/v) yeast extract and 0.5% (w/v) NaCl (Konsoula and Liakopoulou-Kyriakides, 2006). Five mililiter of each overnight strain were used both for immobilization and the free cells experiments.

Immobilization of whole cells in calcium alginate: Immobilization was carried out under sterile conditions. 5 mL of overnight cultures were suspended in 12.5 mL of 2% (w/v) sodium alginate solution (Adinarayana *et al*., 2005). The mixture obtained was extruded drop-wise through a syringe into a 25 mL 3.5% (w/v) $CaCl_2$ solution. Alginate drops were solidified upon contact with $CaCl_2$, forming capsules and thus entrapping the bacterial cells. The capsules (mean volume and diameter 35 μL and 4 mm, respectively) were allowed to harden for 30 min and were then washed with a sterile saline solution (0.9% (w/v) NaCl) to remove any excess Ca^{+2} ions and cells.

Immobilization of whole cells in agar-agar: A definite quantity of agar-agar (Difco) was dissolved in 18 mL of a 0.9% sodium chloride solution to get a final concentration of 2% and was sterilized by autoclaving (Adinarayana *et al*., 2005). The cell suspension (5 mL) was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4-inch Petri plates and allowed to solidify. The solidified agar block was cut into equal size cubes (4 mm^3), added to a sterile 0.1 M phosphate buffer (pH 7.0) and kept in the refrigerator (1 h) for curing. After curing, the phosphate buffer was decanted and the cubes were washed with sterile distilled water three to four times.

Immobilization of whole cells in agarose: Entrapment of cells into agarose was performed by dissolving 1.2 g of agarose in a 20 mL physiological saline at 100°C, autoclaving was conducted and the solution was then cooled to 40-45°C (Adinarayana *et al*., 2005). Subsequently, the agarose solution was mixed with 5 mL cell suspension and poured into sterile flat bottom 4-inch Petri plates and allowed to solidify. The solidified agarose block was cut into equal size cubes (4 mm^3) and kept in the refrigerator for 1 h.

Fermentation: Immobilized cells prepared by the above methods (beads and blocks) were added to 250 mL flasks containing 50 mL Bushnell-Haas (BH) broth medium supplemented with either 2% glucose or 50 mg L^{-1} of tested PAH. For the free cell cultures 50 mL medium was inoculated with bacterial cells equivalent to those used in the immobilized cultures. Batch fermentations with free and immobilized cells were carried out at 30°C for ten days, on a rotary shaker (200 rpm). Samples were taken at regular intervals of two or three days and assayed for biosurfactant production.

Biosurfactant detection: The methylene blue analysis procedure was used for determining the concentrations of biosurfactants in the water samples (Pinzon and Ju, 2009). One mililiter of each sample was first adjusted to pH 2.3±0.2 using 1 N HCl. The acidified samples were then extracted with a five-fold volume of chloroform. Five mililiter of the chloroform extract was carefully removed and put in contact with a freshly prepared methylene blue solution containing 200 μL of methylene blue reagent (1 g L^{-1} methylene blue in 10 mM borax buffer, pH 5.5±0.2) and 4.9 mL of distilled water. Fifteen μL of borax buffer (50 mM sodium tetraborate in distilled water, pH 10.5) was added to the mixture to adjust the pH to 8.6±0.2. The mixture was vigorously mixed for four min and the samples were left to stand for 15 min. The chloroform phase was transferred into a cuvette and the absorbance was measured at 638nm with a Shimadzu UV/Vis spectrophotometer (Model 1601) against chloroform blank. The absorbance values were converted to rhamnolipid concentrations using a calibration curve established by applying the same procedure to standard rhamnolipid solutions of different concentrations.

Bacterial genomic DNA extraction: The genomic DNA of each strain was isolated according to Griffiths *et al.* (2006). Cells were collected from overnight LB cultures by centrifugation and re-suspended in a 500 μL TE buffer. Twenty five microliter of 10 mg mL^{-1} of lysozyme were added and the tubes were incubated at 37°C for 30 min, followed by the addition of 75 μL of 10% SDS and the tubes were inverted gently several times until complete lysis had occurred. Three μL of 20 mg mL^{-1} of Proteinase K were added and the tubes were incubated at 37°C for 1 h. After incubation, 100 μL of NaCl (5M) were added followed by 800 μL of phenol/chloroform: isoamyl alcohol (24:1) and the tubes were inverted several times and then centrifuged for 10 min. The upper phase was transferred to a fresh sterile tube and extracted once with chloroform. The upper phase was again transferred to a fresh tube and a 0.7 volume of isopropanol was added and mixed gently and centrifuged for 10 min. The supernatant was removed carefully and the pellets were washed with 1 mL of 70% ethanol. The pellets were collected by centrifugation for 5 min. The DNA was dried and dissolved in 100 μL TE buffer and stored at -20°C.

Table 1: Code and sequence of the five different random RAPD primers

Oligo name	Sequence
Primer I	5'-d(CCCGTCAGCA)-3'
Primer III	5'-d(GTAGACCCGT)-3'
Primer IV	5'-d(AAGAGCCCGT)-3'
Primer V	5'-d(AACGCGCAAC)-3'
Primer VI	5'-d(GTTTCGCTCC)-3'

Fingerprint using random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis: Fingerprinting using an RAPD-PCR technique was carried out according to (Jabbarzadeh *et al.*, 2010). To detect the molecular variation among the bacterial isolates, P5, N7 and A2, five single arbitrary (10 base) primers were used for RAPD-PCR amplification; their names and sequences are summarized in Table 1. RAPD-PCR was carried out using a Ready-To-Go RAPD analysis kit (Amersham, GE Healthcare, UK). The amplification was performed using a thermocycler (PRIMUS 96) which was programmed as follows; initial denaturation step 95°C for 5 min followed by 35 cycles with 95°C for 1 min for DNA denaturation, annealing at 30°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min, followed by cooling to 4°C. Visualization of amplified DNA fragments was carried out on 2.0% agarose gel in a 1X TBE buffer and stained with ethidium bromide. Moreover, electrophoresis was carried out for 20 min at 150 V and photographed using a gel documentation system (BIO-RAD GeL Doc, 2000).

Data analysis: Patterns of the studied genotypes using RAPD primers were scored as present (1), or absent (0) bands by using the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data. Similarity indices were calculated and a consensus tree was developed based on the obtained banding patterns of the tested genotypes using the SPSS statistical analysis program (Version 10). The genetic relationships among tested genotypes were determined by constructing the phylogenetic tree.

Small-scale preparation of plasmid DNA: The plasmid DNA of each strain was isolated according to Griffiths *et al.* (2006) from overnight LB cultures (16 h). Cells were collected by centrifugation at 12.000 rpm for 30 sec and the supernatant was discarded. The pellets were resuspended in 100 μL of solution I (50 mM glucose,

25 mM Tris.Cl (pH 8.0) and 10 mM EDTA pH 8.0) by vigorous vortexing. Two hundred μL of freshly prepared solution II (0.2 N NaOH (freshly diluted from a 10 N stock and 1% SDS) were added and the contents were mixed by inverting the tubes several times. The tubes were stored on ice for 2 min and 150 μL of ice-cold solution III (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of H_2O) were added. The tubes were gently inverted for 10 s to disperse solution III through the viscous bacterial lysate and the tubes were stored on ice for 3-5 min. The tubes were centrifuged at 12,000 rpm for 5 min and the supernatant was transferred to a fresh tube. The plasmid DNA was precipitated with 0.7 volume of isopropanol, washed with 70% cold ethanol, dried and dissolved in 50 μL of TE buffer (10 mM Tris base (pH 8.0) and 1 mM EDTA pH 8.0).

Amplification and sequencing of the 16S rRNA gene: The 16S rRNA gene was amplified by a Polymerase Chain Reaction (PCR) using specific universal primers (Poppert *et al*., 2005). The sequence for the 16S primer for 16S350F was 5'-AACTGGAGGAAGGTGGGGAT-3' and the sequence for the 16S primer for 16S350R was 5'-AGGAGGTGATCCAACCGCA-3'. The PCR mixture consisted of 30 picomoles of each primer, 10 ng of chromosomal DNA, 200 μM dNTPs and 2.5 units of Taq polymerase with 10 μL of polymerase buffer containing $MgCl_2$. The PCR was carried out for 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. After completion, a fraction of the PCR mixture was examined using 1.5% of agarose gel in a TBE buffer (25 g L^{-1} Tris base, 27.5 g L^{-1} boric acid, 20 mL of 0.5 M EDTA PH 8.0). Electrophoresis was carried out for 20 min at 150 V.

Sequence similarities and phylogenetic analysis: The Blast program (www.blast.ncbi.nlm.nih.gov) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999).

Statistical analysis: Analysis of variance was used to compare the data from the different treatments. All analyses were performed at $p \leq 0.05$ using minitab, version 13.1.

RESULTS AND DISCUSSION

Immobilization is a useful tool to study the ability of a specific microorganism to overproduce specific product at specific conditions. In this study, our bacterial isolates were able to grow and produce biosurfactants in different immobilization matrices. The preferred matrix was chosen according to the type of organism and the type of carbon source.

Production of biosurfactants with immobilized cells in various matrices by entrapment technique using glucose as substrate: Cell immobilization has become one of the most common techniques used for increasing overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. In addition, immobilization of cells may allow continuous operation of cultivation processes at high dilution rates (Beshay *et al*., 2002). Furthermore, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed (Abd-El-Haleem *et al*., 2003).

The applicability of several polymeric natural or synthetic polymers as matrices for immobilization of viable cells motivated the study of the use of different gels such as alginate, agar-agar and agarose. The effects of these types of polymer supports used for immobilization on the biosurfactant production by free and immobilized cells are demonstrated in Fig. 1-3. The tested bacterial strains (P5, N7 and A2) whether free or immobilized were tested for biosurfactant production using BH broth medium supplemented with 2% glucose.

According to the results of this study, the rate of biosurfactant production was high in cases with immobilized cells compared with free cells. However, the three strains tested showed different production rates with a different preferable matrix. For example, P5 strain (Fig. 1) showed the highest biosurfactant production rate when immobilized with alginate (136 mg L^{-1}), followed by

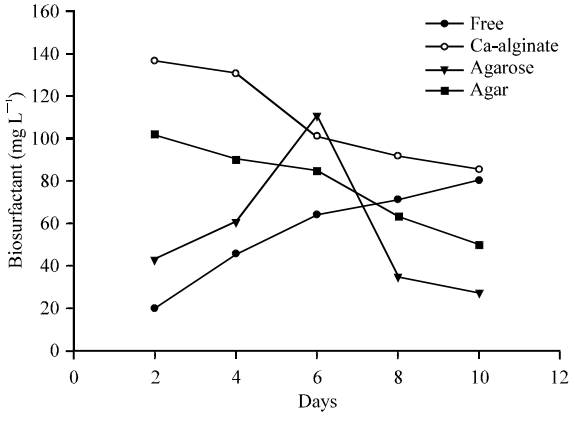

Fig. 1: Effect of different immobilization matrices on biosurfactant production of P5 isolate grown at BH medium supplemented with 2% glucose

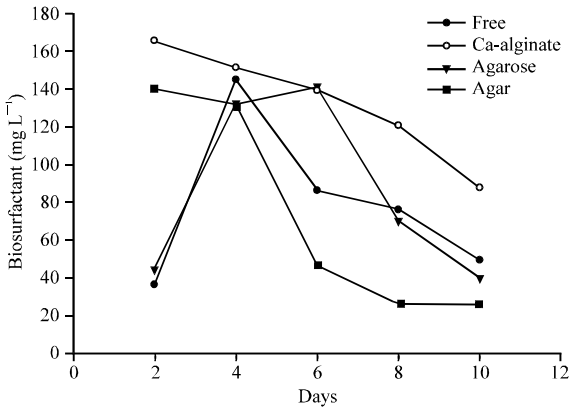

Fig. 2: Effect of different immobilization matrices on biosurfactant production of N7 isolate grown at BH medium supplemented with 2% glucose

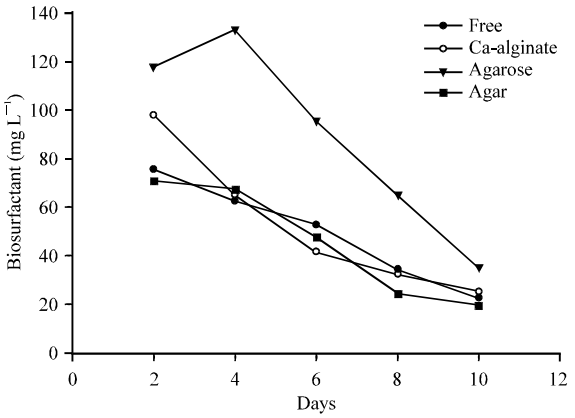

Fig. 3: Effect of different immobilization matrices on biosurfactant production of A2 isolate grown at BH medium supplemented with 2% glucose

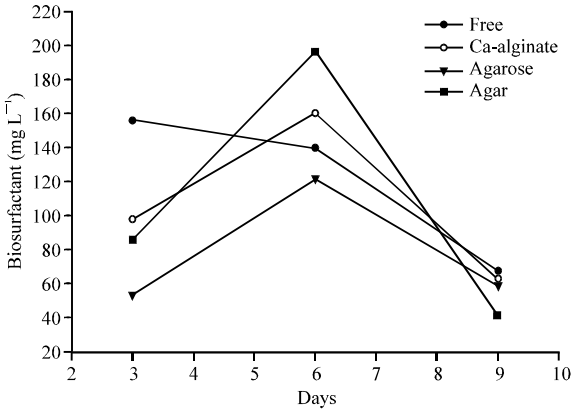

Fig. 4: Production of biosurfactant using different immobilization matrices using P5 isolate grown at BH medium+50 mg L^{-1} phenantherene

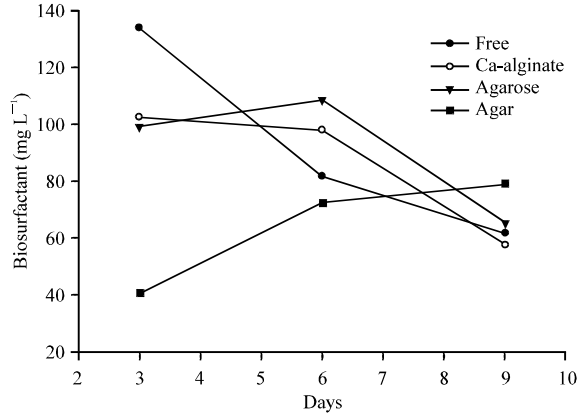

Fig. 5: Production of biosurfactant using different immobilization matrices using N7 isolate grown at BH medium+50 mg L^{-1} naphthalene

agar (110 mg L^{-1}) and then agarose (101 mg L^{-1}). While in cases with the N7 isolate (Fig. 2), the highest biosurfactant concentration was obtained when the cells were immobilized with alginate (165.3 mg L^{-1}) followed by agarose (141 mg L^{-1}) and then agar gels (140 mg L^{-1}). The immobilization of the A2 strain with agarose gel displayed the highest production rate of biosurfactant (132.4 mg L^{-1}) compared to alginate and agar (97.4 and 70.6 mg L^{-1}, respectively). Therefore, it can be concluded that the alginate gel showed higher production efficiency than the other two gels. This might be due to the differences in the porous structures of the matrices which allow a better growth of the cells in the alginate matrix (Dias *et al*., 2000) and thus a higher production of biosurfactant.

Production of biosurfactants with immobilized cells in various matrices by entrapment technique using PAHs as substrate: The effect of different immobilization matrices on biosurfactant production was investigated in the presence of PAH as the sole source of carbon and energy. The tested bacterial isolates (P5, N7 and A2) were immobilized in different matrices including alginate, agarose and agar. The immobilized microorganisms were then grown in BH medium supplemented with 50 mg L^{-1} of the tested hydrocarbons (anthracene, naphthalene and phenanthrene). The three bacterial isolates showed different production levels of biosurfactant according to the matrices used in cell immobilization (Fig. 4-6). In the case of agar immobilized cells, isolates P5 and A2 produced a high amount of biosurfactant (195.9 and

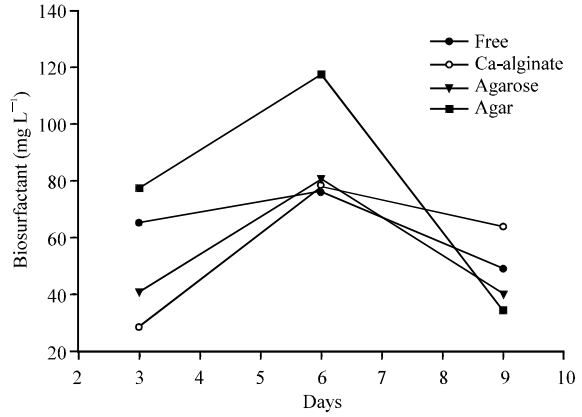

Fig. 6: Production of biosurfactant using different immobilization matrices using A2 isolate grown at BH medium supplemented with 50 mg L^{-1} anthracene

121 mg L^{-1}, respectively), whereas the free cells of isolate N7 produce a higher amount of biosurfactant (133.7 mg L^{-1}) compared with immobilized cells.

It is assumed that the high production level of biosurfactant obtained by the agar immobilized cells of isolates P5 and A2 may be due to the fact that the agar caves protect the bacterial cells from the toxicity of the PAH residue (Adinarayana *et al*., 2005). This kind of protection enables the bacterial cells to produce the biosurfactant that directly facilitates the availability of PAH to be consumed by the cells. We believe that agar immobilized cells of isolates P5 and A2 produced a high amount of biosurfactant when they utilized the phenanthrene and anthracene as the sole carbon source. However, a high production of biosurfactant was obtained when N7 bacterial cells were free and grown on naphthalene. This might be due to the fact that naphthalene is more readily available in the medium inoculated with free cells but this availability decreased when the cells were immobilized. This result is in harmony with the findings of other researchers who have suggested that immobilized biocatalysts produce lower levels of enzyme in comparison to free cells due to diffusion barriers and reduced oxygen availability for immobilized aerobic cells (Mamo and Gessesse, 1997; Konsula and Liakopoulou-Kyriakides, 2004)

Molecular identification of the three selected bacterial isolates

RAPD-PCR analysis: The RAPD-PCR technique was used to differentiate between the three selected isolates P5, A2 and N7 using five arbitrary primers. Different band patterns were observed with each primer (Fig. 7). Notably, high similarity was observed between A2 and N7. The RAPD results revealed that about 107 reproducible fragments were obtained and a total of 51 bands out the 107 were monomorphic (47.6%) and the rest were polymorphic. Similar studies were performed to study the genetic diversity between different bacterial species. For example, Istock *et al*. (2001) used RAPD-PCR for studying the geographical diversity of genomic lineages in *Bacillus subtilis*. In addition, Drake *et al*. (1996) used the single primer G1 that could distinguish 16 commercial strains of *Lactobacillus helveticus* by RAPD-PCR profiling. Moreover, Matar *et al*. (2009) used RAPD-PCR to study genetic diversity among 14 antagonistic *Bacillus subtilis* isolates obtained from different Egyptian sites. Josic *et al*. (2008) have also reported the ability of RAPD fingerprinting to differentiate between the two isolates of *Lysinibacillus fusiformis* obtained from Oil-Polluted Soil. In addition, Michelim *et al*. (2008) studied the molecular epidemiology of infection due to *Proteus* species using a variety of methods, including RAPD-PCR. They reported that the RAPD technique is one of the most suitable methods for local *Proteus* epidemiological studies. Moreover, Abdo-Hasan *et al*. (2008) have used the RAPD technique to differentiate between 40 isolates of *Xanthomonas axonopodis* pv. *malvacearum* the causal agent of cotton angular leaf spot disease. They demonstrated that the RAPD technique revealed high degrees of polymorphisms among the studied races. Pooler *et al*. (1996) used three PCR-based techniques which rely on different amplification priming strategies, RAPD, Repetitive Extragenic Palindromic (REP) and Enterobacterial Repetitive Intergenic Consensus (ERIC), to study the genetic relationships among 25 isolates of *Xanthomonas fragariae* from diverse geographic regions. In conclusion, unlike biochemical and serological methods, molecular tools for genotyping strains are more accurate in terms of identifying sub-lineages of microorganism (Kumar *et al*., 2007).

According to the data obtained using the RAPD-PCR technique, all the primers succeeded to distinguish between the three isolates. Data shown in Fig. 8 revealed that the three isolates were grouped into two main groups. The first group contained isolates A2 and N7 which are highly similar to each other. The second group contained only the P5 isolate which showed less similarity with the other two isolates. These results indicated that the P5 isolate belongs to a genus that is completely different from the genus of the A2 and N7 isolates.

Plasmid profile of the three bacterial isolates: Plasmids are extrachomosomal circular double stranded DNA found in most bacteria. Each bacterium may

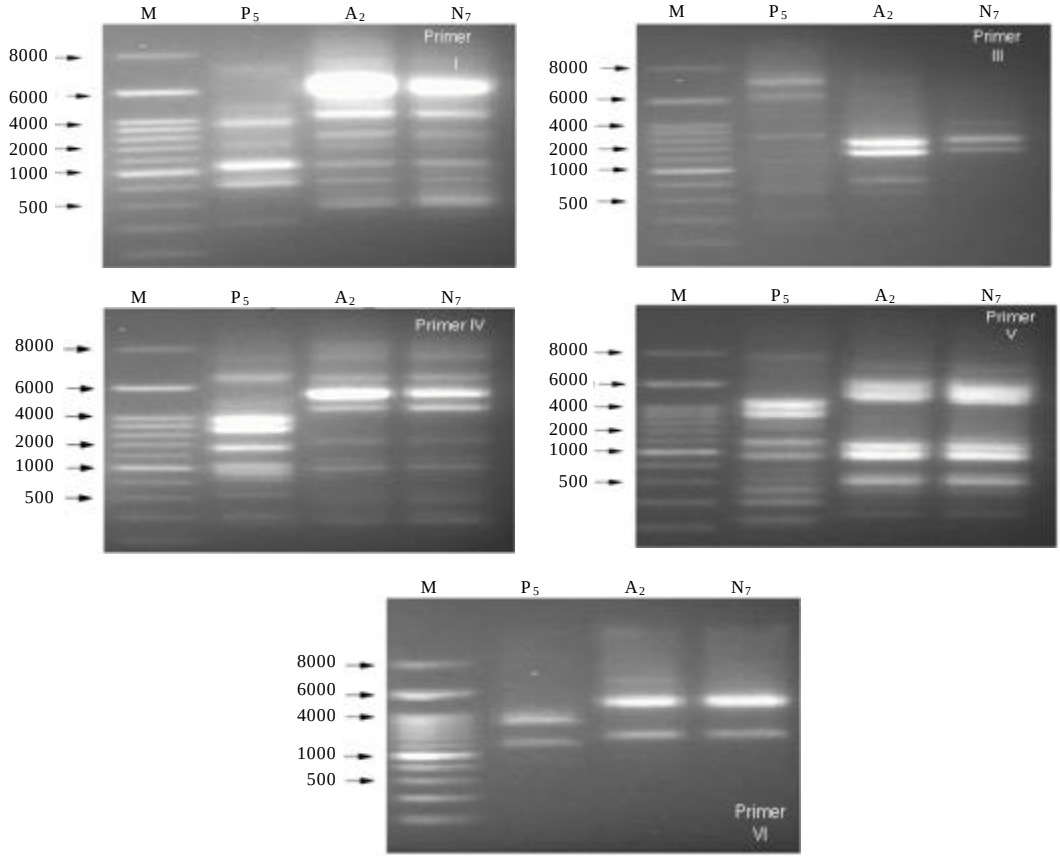

Fig. 7: RAPD-PCR banding patterns for the three bacterial genotypes using primer I, III, IV, V and VI, M: 1 kb ladder, P5, A2 and N7: Bacterial isolates

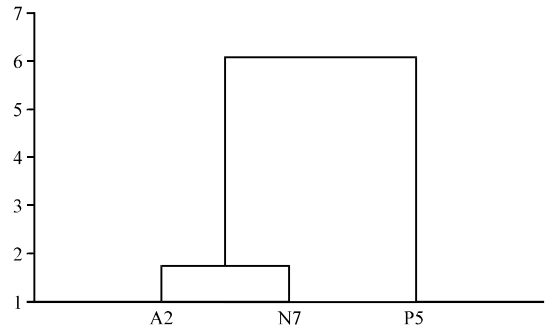

Fig. 8: Consensus tree for three bacterial genotypes developed on the basis of their banding patterns with five RAPD primers

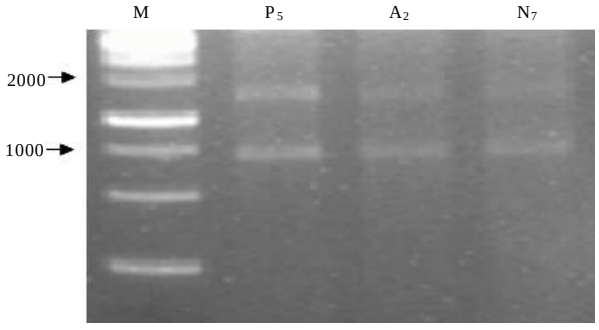

Fig. 9: Plasmid profile of the isolates P5, A2 and N7, M: 1 kbp marker, P5, A2 and N7: Bacterial isolates

contain one or several plasmids. Plasmid profile analysis involves the study of the size and number of plasmids (Busch and Nitschko, 1999). This gives the size and number of plasmids present in the cells. Some species may contain a variable number of plasmids and even unrelated bacteria may harbor a similar number of plasmids (Li *et al*., 2002). In this study, two different plasmids were detected in the three bacterial isolates P5, N7 and A2 (Fig. 9). Accordingly, the estimated molecular sizes of the two

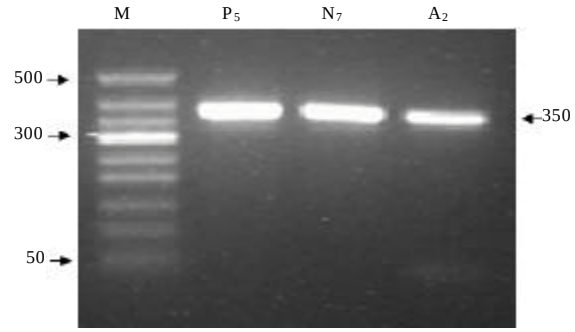

Fig. 10: PCR amplification for the 16S rRNA gene from the three selected bacterial isolates degrading the polyaromatic hydrocarbons, M: 50 bp ladder

plasmids were 2 and 1 kbp, respectively. It was noticed that, the two isolated molecular weight plasmids were presented in the three tested isolates.

Since, plasmid profile analysis is one of the methods used for differentiation between bacterial strains, many authors have investigated the ability of plasmid profile analysis to discriminate bacterial strains from each other. Appuhamy *et al*. (1998) have reported that plasmid profile analysis has added a further level of discrimination between *Histophilus ovis* strains and is a useful supplement to the PCR typing methods. In addition, it was demonstrated that plasmid profile typing is a useful method for the rapid differentiation of *Salmonella enteritidis* phage type 4 strains isolated from humans, poultry and eggs (Threlfall *et al*., 1994). The plasmid profile in this investigation was not suitable to discriminate between the three tested isolates. This might be due to the fact that the number of examined strains was not large enough to give a considerable variation.

Molecular identification of the selected bacterial isolates using 16S rRNA gene: For bacterial identification, the genomic DNA was subjected to PCR amplification using universal primers. These primers were able to amplify about 350 bp from the 16S rRNA gene (conservative region). The PCR product was separated on 1.5% agarose gel and data presented in Fig. 10 reveals that amplicone with a molecular size 350bp were observed with the three examined bacterial isolates.

The result of 16S rRNA gene alignment of the three isolates with the Gene Bank revealed the P5 isolate was identified as *Lysinibacillus fusiformis* with a similarity of 98%, whereas N7 and A2 were identified as *Cronobacter turicensis* with 99% similarity. The 16S rRNA sequences of the bacterial isolates were deposited

Table 2: Strain codes, strain name, accession number and similarity percentage of experimental isolates

Strain code	Strain name	Accession No.	Similarity (%)
P5	*Lysinibacillus fusiformis* EHTH1	JN181395	98
N7	*Cronobacter turicensis* EHTH2	JN181397	99
A2	*Cronobacter turicensis* EHTH3	JN181396	99

in Gene Bank (NCBI). The accession number of each isolate and percentage similarity to Gene Bank deposited strains are shown in Table 2. The bacterial isolate *Lysinibacillus fusiformis* strain had been previously isolated from oil polluted soil and showed an ability to degrade toluene, xylole and crude oil (Josic *et al*., 2008) in addition to other different hydrocarbons (Bento *et al*., 2003).

CONCLUSION

This study demonstrates that PAH degrading bacterial isolates can produce biosurfactant as a potent method to overcome bioavailability problem. In addition, the bacterial isolates prefer to be immobilized than to be free during the degradation process. In addition, the type of preferred immobilization matrix depends on the used carbon source; where, in general, calcium alginate was preferred at the presence of glucose while agar was preferred at the presence of hydrocarbons.

REFERENCES

Abd-El-Haleem, D., U. Beshay, A. Abdelhamid, H. Moawad and S. Zaki, 2003. Effects of mixed nitrogen sources on biodegradation of phenol by immobilized *Acinetobacter* sp., strain W-17. Afr. J. Biotechnol., 2: 8-12.

Abdo-Hasan, M., H. Khalil, B. Debis and N. MirAli, 2008. Molecular characterization of syrian races of *Xanthomonas axonopodis* pv. *Malvacearum*. J. Plant Pathol., 90: 431-439.

Adinarayana, K., B. Jyothi and P. Ellaiah, 2005. Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. APPS Pharm. Sci. Tech., 6: 391-397.

Appuhamy, S., J. Low, J. Coote and R. Parton, 1998. PCR methods and plasmid profile analysis for characterization of *Histophilus ovis* strains. J. Med. Microbiol., 47: 987-992.

Bento, F., F. Camargo, B. Okeke and W. Frankenberger-Junior, 2003. Bioremediation of soil contaminated by diesel oil. Braz. J. Microbol., 34: 65-68.

Beshay, U., D. Abd-El-Haleem, H. Moawad and S. Zaki, 2002. Phenol biodegradation by free and immobilized *Acinetobacter*. Biotechnol. Lett., 24: 1295-1297.

Busch, U. and H. Nitschko, 1999. Methods for the differentiation microorganisms. J. Chromatogra. B. Biomed. Sci. Appl., 722: 263-278.

Darrabie, M., W. Kendall and E. Opara, 2006. Effect of alginate composition and gelling cation on micro-bead swelling. J. Microencapsul., 23: 29-37.

Dias, J.C.T., R.P. Rezende and V.R. Linardi, 2000. Biodegradation of acetonitrile by cells of *Candida guilliermondii* ufmg-y65 immobilized in alginate, k-carrageenan and citric pectin. Braz. J. Microbol., 31: 61-66.

Drake, M.A., C.L. Small, K.D. Spence and B.G. Swanson, 1996. Differentiation of *Lactobacillus helveticus* strains using molecular typing methods. Food Res. Int., 29: 451-455.

Feijoo-Siota, L., F. Rosa-Dos-Santos, T. de-Miguel and T.G. Villa, 2008. Biodegradation of naphthalene by *Pseudomonas stutzeri* in marine environments: Testing cell entrapment in calcium alginate for use in water detoxification. Bioremediat. J., 12: 185-192.

Gentili, A.R., M.A. Cubitto, M. Ferrero and M.S. Rodriguez, 2006. Bioremediation of crude oil polluted seawater by a hydrocarbon degrading bacterial strain immobilized on chitin and chitosan flakes. Int. Biodeter. Biodegrad., 57: 222-228.

Griffiths, L.J., M. Anyim, S.R. Doffman, M. Wilks, M.R. Millar and S.G. Agrawal, 2006. Comparison of DNA extraction methods for *Aspergillus fumigates* using real-time PCR. J. Med. Microbol., 55: 1187-1191.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid Symp. Ser., 41: 95-98.

Holman, H.Y.N., Y.W. Tsang and W.R. Holman, 1999. Mineralization of sparsely water-soluble polycyclic aromatic hydrocarbons in water table fluctuation zone. Environ. Sci. Technol., 33: 1819-1824.

Istock, C.A., N. Ferguson, N.L. Istock and K.E. Duncan, 2001. Geographical diversity of genomic lineages in *Bacillus subtilis* (Ehrenberg) Cohn sensu lato. Org. Divers. Evol., 1: 179-191.

Jabbarzadeh, Z., M. Khosh-khui, H. Salehi and A. Saberivand, 2010. Inter Simple Sequence Repeat (ISSR) markers as reproducible and specific tools for genetic diversity analysis of rose species. Afr. J. Biotech., 9: 6091-6095.

Josic, D., M. Porobic, M. Milicevic, D. Vukovic, R. Pivic, M. Zdravkovic and T. Coric, 2008. RAPD fingerprinting of indigenous *Lysinibacillus fusiformis* isolates from stabilized sludge and oil-polluted soil. Proceedings of the International Meeting on Soil Fertility Land Management and Agroclimatology, October 29-November1, 2008, Kusadasi, Turkey, pp: 927-933.

Konsoula, Z. and M. Liakopoulou-Kyriakides, 2006. Thermostable α-amylase production by *Bacillus subtilis* entrapped in calcium alginate gel capsules. Enzyme Microb. Technol., 39: 690-696.

Konsula, Z. and M. Liakopoulou-Kyriakides, 2004. Hydrolysis of starches by the action of the α-amylase from *Bacillus subtilis*. Process Biochem., 39: 1745-1749.

Kumar, A.R., V. Sathish, G.B. Nair and J. Nagaraju, 2007. Genetic characterization of *Vibrio cholera* strains by inter simple sequence repeat-PCR. FEMS Microbiol. Lett., 272: 251-258.

Li, M.S., Y.H. Je, I.H. Lee, J.H. Chang and J.Y. Roh *et al*., 2002. Isolation and characterization of a strain of *Bacillus thuringiensis* sp. kurstaki containing a new delta-endotoxin gene. Curr. Microbiol., 45: 299-302.

Lim, L.H., R.M. Harrison and S. Harrad, 1999. The contribution of traffic to atmospheric concentrations of polycyclic aromatic hydrocarbons. Environ. Sci. Technol., 33: 3538-3542.

Makkar, R.S. and K.J. Rockne, 2003. Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem., 22: 2280-2292.

Mamo, G. and A. Gessesse, 1997. Thermostable amylase production by immobilized *Bacillus* sp. Biotechnol. Tech., 11: 447-450.

Manohar, S., C.K. Kim and T.B. Karegoudar, 2001. Enhanced degradation of naphthalene by immobilization of *Pseudomonas* sp. strain NGK1 in polyurethane foam. Appl. Microbiol. Biotechnol., 55: 311-316.

Matar, S.M., S.A. El-Kazzaz, E.E. Wagih, A.I. El-Diwany and M.A. El-Saadani *et al*., 2009. Molecular characterization and batch fermentation of *Bacillus subtilis* as biocontrol agent, II. Biotechnology, 8: 35-43.

Michelim, L., G. Muller, J. Zacaria, A.P. Delamare, S.O. Costa and S. Echeverrigaray, 2008. Comparison of PCR-Based molecular markers for the characterization of *Proteus mirabilis* clinical isolates. Braz. J. Infect. Dis., 12: 423-429.

Moslemy, P., R.J. Neufeld and S.R. Guiot, 2002. Biodegradation of gasoline by gellan gum-encapsulated bacterial cells. Biotechnol. Bioeng., 80: 175-184.

Mrozik, A., Z. Piotrowska-Seget and S. Labuzek, 2003. Bacterial degradation and Bioremediation of polycyclic aromatic hydrocarbons. Pol. J. Environ. Stud., 12: 15-25.

Phillips, T.M., D. Liu, A.G. Seech, H. Lee and J.T. Trevors, 2000. Monitoring bioremediation in creosote-contaminated soils using chemical analysis and toxicity tests. J. Indust. Microbio. Biotech., 24: 132-139.

Pinzon, N.M. and L.K. Ju, 2009. Analysis of rhamnolipid Biosurfactants by methylene blue complexation. Applied Microbiol. Biot., 82: 975-981.

Piskonen, R. and M. Itavaara, 2004. Evaluation of chemical pretreatment of contaminated soil for improved PAH bioremediation. Appl. Microbiol. Biotechnol., 65: 627-634.

Pooler, M.R., D.F. Ritchie and J.S. Hartung, 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random polymorphic DNA PCR, repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. Appl. Environ. Microbiol., 62: 3121-3127.

Poppert, S., A. Essig, B. Stoehr, A. Steingruber and B. Wirths *et al*., 2005. Rapid diagnosis of bacterial meningitis by real-time PCR and fluorescence in situ hybridization. J. Clin. Microbiol., 43: 3390-3397.

Rockne, K.J., L.M. Shor, L.Y. Young, G.L. Taghon and D.S. Kosson, 2002. Distributed sequestration and release of PAHs in weathered sediment. The role of sediment structure and organic carbon properties. Environ. Sci. Technol., 36: 2636-2644.

Ron, E.Z. and E. Rosenberg, 2002. Biosurfactants and oil bioremediation. Curr. Opin. Biotech., 13: 249-252.

Ruma, R., R. Ray, R. Chowdhury and P. Bhattacharya, 2007. Degradation of polyaromatic hydrocarbons by mixed culture isolated from oil contaminated soil-A bioprocess engineering study. Indian J. Biotech., 6: 107-113.

Seoud, M.A and R. Maachi, 2003. Biodegradation of naphthalene by free and alginate entrapped *Pseudomonas* sp. J. Biosci., 58: 726-731.

Sergio, A.M. and T.Y. Bustos, 2009. Biodegradation of wastewater pollutants by activated sludge encapsulated inside calcium-alginate beads in a tubular packed bed reactor. Biodegrad., 20: 709-715.

Tao, X., G. Lu, J. Liu, T. Li and L. Yang, 2009. Rapid degradation of phenanthrene by using Sphingomonas sp. GY2B immobilized in calcium alginate gel beads. Int. J. Environ. Res. Public Health, 6: 2470-2480.

Tao, X.Q., J.P. Liu, G.N. Lu, X. Guo, H.P. Jiang and G.Q. Sun, 2010. Biodegradation of phenanthrene in artificial seawater by using free and immobilized strain of *Phingomonas* sp. GY2B. Afr. J. Biotechnol., 9: 2654-2660.

Threlfall, E.J., M.D. Hampton, H. Chart and B. Rowe, 1994. Use of plasmid profile typing for surveillance of *Salmonella enteritidis* phage type 4 from humans, poultry and eggs. Epidemiol. Infect., 112: 25-31.

Timmis, K.N. and D.H. Pieper, 1999. Bacteria designed for bioremediation. Trends Biotechnol., 17: 200-204.

Ward, O., A. Singh and J.V. Hamme, 2003. Accelerated biodegradation of petroleum hydrocarbon waste. J. Ind. Microbiol. Biotechnol., 30: 260-270.