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BIODEGRADTION AND BIOSTRIPPING OF POLYURETHANE COATINGS BY HYDROCARBON-DEGRADING BACTERIA

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ABSTRACT

Five locally isolated strains SBI (1-5) were cultivated on mineral salts-agar with polyurethane as the sole carbon and energy source. All the tested microorganisms grew on polyurethane except SBI-4. However, the growth inhibition of SBI-4 was temporary. Except of SBI-4, all microogrganisms grew and prossessed esterase activity when cultured on mineral salts medium containing polyurethane or on an enriched medium. When SBI-5 isolate was allowed to grew in mineral salts liquid culture media containing polyurethane-painted aluminum coupons, a change in color as well as an increase of total protein and optical density of the culture broth were evidences for biostripping of the polyurethane coating. Immobilization of SBI-5 cells on polyurethane-coated coupons with the aid of a thin layer of calcium alginate showed more stripping of the dark colored coating of the aluminum coupons than that obtained with free suspended cells. Scanning electron microscopy of microbially treated polyurethane painted coupons revealed a reduction of the originally hillet's surfaces.

INTRODUCTION

Biodergradation of polyurethane (PU) was established by an early investigation[1]. Later work[2] showed that polyester PUs are more susceptible to biodegradation than polyether PUs by fungi. Several investigators[3,4] reported about degradation in marine environments and the sequence of attack by mixed organisms on various functional groups present in PUs. The role of extracellular enzymes in biodegradation of polyurethane[5,6] was demonstrated. IR spectroscopy and thin layer chromatography were able to monitor the growth of Penicillium citrinum (a fungus known to produce a polyester-degrading esterase) on PU[7]. The same investigators also studied the attack of Gliocladium roseum and Chaetomium globosum on PUs, where they observed that the use of a complete culture medium delayed microbial utilization of PU compared to a minimal basal salts medium. Cryptococcus laurentii and Pseudomonas aeruginosa are known to hydrolyze polyurethanes by the action of their exoenzymes[8].

Biosurfactants have been observed to play important roles in many biodegradation processes, although their role in polyurethane biodegradtion has apparently yet to be observed. Biosurfactants could be important additions to biostripping formulations. Biological surfactants from Ps. aeruginosa were used for removing oil from Alaskan gravel contaminated by the Exxon Valdex oil spill[9]. Microbial surfactant released oil 2 to 3 times more extensively than warm water.

Recently, a good correlation between biodegradative activity of a microorganism of natural heavy hydrocarbons and its ability to produce a biosurfactant was demonstrated[10]. A locally isolated Arthrobacter globiformis SBI-5 showed a remarkable biodegradative activity for heavy hydrocarbons[10].

This paper deals with screening of the capability of 5 different bacterial strains to utilize PU as their sole source of carbon and energy in mineral salts medium. Moreover, the relationship between esterase activity of these strains and their growth on PU, as well as biostripping of a PU coating from aluminum coupons by the most active strain (SBI-5) was investigated.

EXPERIMENTAL

Microorganisms:

Five locally isolated (SBI 1-5)[10] were used through this investigation. For propagation, pure microbial strains were subcultured on Trypticase-Soy-Agar (TSA) slants and incubated at 30°C for 24-48 hours. The grown cells were stored at 4°C and renewed every 4 weeks.

Culture media:

In addition to nutrient agar, nutrient broth, Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were also used. Minimal salts medium, MSB-1, and its supplements constituents was most widely used and was prepared as previously described[10,11].

Preparation of Bacterial Suspension:

Cells from actively growing slants were harvested and suspended in saline solution (9 g/L NaCl) to produce an OD_{546} of 1.0. An aliquot of 1 ml of this suspension was added to 50 ml of TSB (DIFCO), in 125 ml Erlenmeyer flasks and incubated at 30°C for 24 hours in a shaking water bath (150 rpm). The cells were harvested by centrifugation and resuspended in sterile demineralized water to give OD_{546} of about 1.0. This suspension was then used to inoculate the S-MSB-1 media in test cultures.

Biodegradation of polyurethane "Liquid Plastic" in MSB-1 with SBI-5 cells:

Glass slides were carefully cleaned with nitric acid, washed several times with distilled water and dried. The glass slides were then coated with polyurethane paint (Final Touch, United Coating, Inc., Polyurethane Liquid Plastic, USA). After drying, the slides were sterilized by soaking in 70% (v/v) ethanol for 30 min. at room temperature.

Under sterile conditions two plastic containers for holding glass slides were half-filled with a suspension of Arthrobacter globiformis, SBI-5 cells in MSB-1 medium. Polyurethane-coated glass slides were placed on edge in the slides boxes. Lids were placed on the boxes and setrile air was sparged through the culture broth while the cultures were kept at room temperature. The broth cultures were analyzed by determining optical density, pH, and total and soluble protein. Periodically, the polyurethane coated glass slides were examined microscopically.

Biostripping of polyurethane paint:

Preparation of polyurethane-painted coupons:

Aluminum flashing was cut into 2.5 x 3.2 cm rectangles. A handle was formed by inserting aluminum wire through the drilled hole near the edge and twisting the wire upon itself to form a closed loop. The coupons were cleaned, primed and painted with gray colored polyurethane paint.

Growing microorganisms on solid agar with polyurethane as the sole carbon and energy source:

The above mentioned five test bacteria were screened for their ability to degrade polyurethane paint. These microor-

ganisms were cultured on MSB-1-agar plates containing polyurethane-painted metal coupons (prepared as described above) as a sole carbon and energy source. Purified Agar Agar (Difco) was dissolved in MSB-1 medium in a concentration of 1.5% (w/v). After sterilization by autoclaving, exactly 15 ml of the hot agar liquid (50-55°C) was inoculated with 150 µl of microbial suspenion (with OD_{546} of about 1) in physiological saline (9 g NaCl/L), mixed gently and poured in a sterile Petri dish. The plates were left overnight to dry at room temperature before using. A sterilized polyurethane-coated aluminum coupon was laid flat onto the surface of freshly prepared plain agar, then covered with a second layer of agar containing a suspenion of the test microorganism under aseptic conditions. All inoculated plates were incubated at 30°C and examined daily for growth. As a negative growth control, the above described MSB-1-agar plates inoculated with microorganisms were used, but without polyurethane-coated coupons. As a positive growth control, the same suspenion of the above used microorganisms were streaked on the surface of nutrient agar plates without polyurethane.

Biodegradation of polyurethane paint by Arthrobacter globiformsi SBI-5 in submerged culture:

Aluminum wires attached to the polyurethane-painted coupons were pushed through rubber stoppers that fit the mouth of 500 ml Erelenmeyer flasks. The rubber stoppered flasks with ten polyurethane-coated coupons hanging from the rubber stopper and suspended approximately one centimeter above the bottom of the flasks were sterilized by autoclaving at 121°C for 20 minutes. Freshly sterilized medium (S-MSB-1) (250 ml per flask) was added to the sterile 500 ml Erelenmeyer flask with polyurethane-coated coupons. The fermentation medium was then inoculated with a freshly prepared suspenion of SBI-5 cells to give at zero time an OD_{546} of about 0.1. The cultures were then incubated at 30°C in a rotary water bath agitated at 150 rpm. Sterile air was sparged directly through the culture broth. After 168 hours of fermentation, the culture broth of both cultures was carefully removed and replaced with fresh, sterilized S-MSB-1 medium to begin a second fermentation cycle which lasted an additional 144 hours. Total protein concentration in the culture broth was determined. Changes in the pH of the culture broth were monitored. Deterioration of the polyurethane coating was accessed visually.

Immobilization and the culture conditions of SBI-5 cells on the polyurethane-painted coupons:

Sodium alginate (4.5 grams) was suspended in 125 ml of demineralized water and dissolved by heating. After cooling the alginate solution to room temperature, 25 ml of microbial cell suspension was added to the alginate solution. This alginate-cell slurry were then used to coat six polyureythane-painted, aluminum coupons. The coupons were dipped into the alginate-cell slurry under aseptic conditions for five minutes. The coupons were then transferred to a 2% $CaCl_2$ for one hour. The alginate-covered, polyurethane-coated coupons were washed with sterile demineralized water. The coupons were submerged in 300 ml of freshly sterilized S-MSB-1 in a 500 ml Erelenmeyer flask. As a control, a second Erlenmeyer flask with 300 ml of freshly sterilized S-MSB1 and six polyurethane-painted coupons were inoculated with three ml of the bacterial suspension. Cultures were incubated at 30°C in a shaker water bath gyrating at 150 rpm. Sterile air was sparged directly through the culture broths. After 14 days an additional 150 ml of freshly sterilized S-MSB1 medium was added to both cultures. The cultures were then incubated for an additional 16 days (total fermentation time 30 days).

Analysis:

Determination of cell growth:

Total protein concentration in the culture broth was determined spectro-

photometrically using Bovine Serum Albumin (BSA) as the standard[12].

Esterase activity:

Fluorescein diacetate was used as an indicator of esterase activity. Esterase cleaves the acetate from fluorescein diacetate producing the fluorophore fluorescein. In this method, agar discs (1 cm in diameter) containing microorganisms were cut from the solid agar covering the polyurethane-coated coupons. The discs of agar were placed on the surface of freshly prepared mineral salts agar plates containing 20 mg/L fluorescein diacetate. After incubation at 30°C, the Petri dishes were viewed under ultra-violet light every 24 hours. A qualitative assessment of esterase activity was based on fuoresecent intensity.

Scanning electron microscopy of polyurethane-coated coupons:

Polyurethane-coated aluminum coupons obtained from submerged cultures and preserved in 5% glutaraldehyde were examined by scanning electron microscopy (SEM). One polyurethane-painted coupon not used in the fermentation experiments was used as a control. Prior to examining the coupons by SEM, they were gently, rinsed in water and 0.1 M cacodylate, treated with 10% osmium tetroxide (w/v) for 30 minutes, rinsed with water, dehydrated in a graded series of ethanol solutions, critical point dried with liquid CO_2, mounted on SEM stubs with silver paint, and then sputter coated with gold for two minutes.

RESULTS AND DISCUSSION

Growing microorganisms on solid agar with polyurethane as the sole carbon and energy source:

Signs of growth in the cultures of Acinetobacter calcoaceticus (SBI-1), Pseudomonas sp. (SBI-2), Pseudomonas sp. (SBI-3), and Arthrobacter globiformis (SBI-5) cultures were observed. The growth appeared to be localized predominately in the locales of the coupons. In contrast, no sign of growth was noted in the culture of Pseudomonas cepacia (SBI-4). However, when a plug of an inoculated agar from SBI-4 culture containing a polyurethane-coated coupon was subcultured onto nutrient agar, active growth of the subcultured SBI-4 was observed. This indicates that the microogranism remained viable and retained its growth potential even though it was unable to utilize polyurethane to support growth.

Growing microorganisms on solid agar with polyurethane in the presence of fluorescein diacetate as an esterase indicator:

Since, it is likely that esterase is an important enzyme in the biodegradation of polyurethane[7], experiments were performed to determine which of the test organisms possesses esterase activity. All test microbes taken from cultures containing polyurethane and control microbes (grown on nutrient agar) possessed esterase activity, except SBI-4. The results show that esterase is a constitutive enzyme in all microorganisms which showed esterase activity.

Biodegradation of polyurethane-painted aluminum coupons by SBI-5 in submerged cultures:

Figure 1, shows the optical densities and the protein concentrations of SBI-5 culture broths during the first and the second fermentation cycles. Based on the total protein profiles for SBI-5, it appears that the organism grew on polyurethane as sole carbon and energy source efficiently for 12 days in two fermentation cycles. There is no correlation between the increase in the total protein content and the cell mass yield, specially in the first fermentation cycle. It was noticed, however, that as fermentation proceeded forward, the dark gray pigment from deteriorated polyurethane coating became suspended in the culture broth. A portion of the increased absorbance of this culture broth may be caused by the pigment that becomes suspended therein.

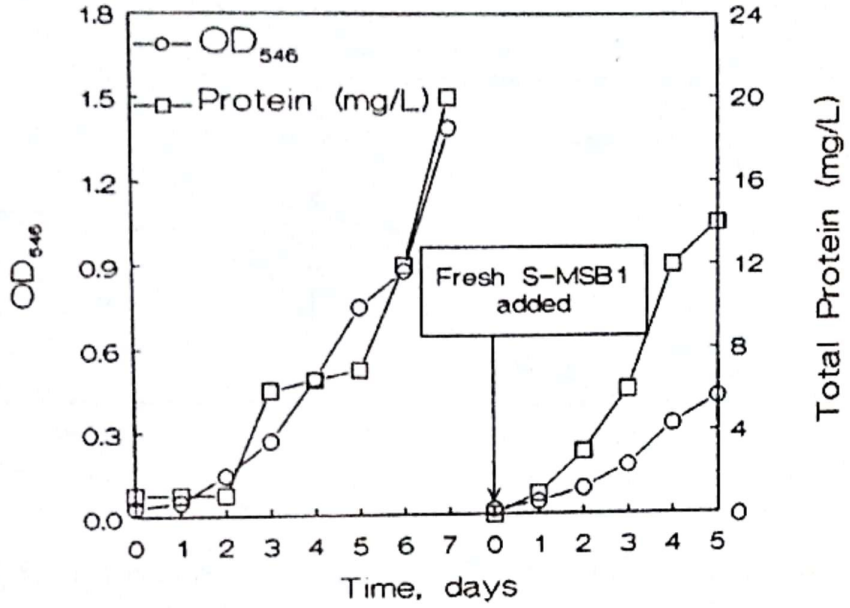

Figure (1): Growth profiles (OD_{546}) and total protein (mg/L) of Arthrobacter globiformis SBI-5 cultures using liquid plastic polyurethane coated to glass slides.

The change in color of the broth to the gray reached its maximum within 64 to 96 hours of culturing. However, this change was less during the second growth cycle than the first growth cycle where there was no obvious discoloration of the polyurethane coatings on the coupons themselves at the end of the fermentation. It is further important to note that the coupon's coating appeared to remain intact and the paint did not delaminate from the coupons.

Immobilization of SBI-5 at the polyurethane-painted aluminum coupon's surface:

Since, free SBI-5 cells degrade polyurethane coating on metal coupons, the possibility of increasing biostripping by concentrating cells at or near the polyurethane surface by immobilization was investigated. The production of pigmented sediment released from the polyurethane coating was followed visually. Figures 2 and 3 compare the OD_{546} and the protein concentration of the broth from polyurethane cultures with SBI-5 cells suspended freely in the culture broth and those immobilized within alginate coatings on polyurethane-painted coupons. During the first 14 days of fermentation, the free-cell culture broth possessed higher total protein concentrations than the culture broth of the immobilized cells. In contrast, there was little difference in the OD_{546} of the two culture broths. This suggets either that: 1) there are fewer cells in the immobilized cell culture: or 2) there may be equal or even more number of cells remained trapped in the alginate-coated coupons.

Since, many samples of the broth had been removed from both cultures (free and immobilized) during the 14 days culture period, 150 ml of freshly prepared S-MSB-1 medium were added to each culture. The culture containing

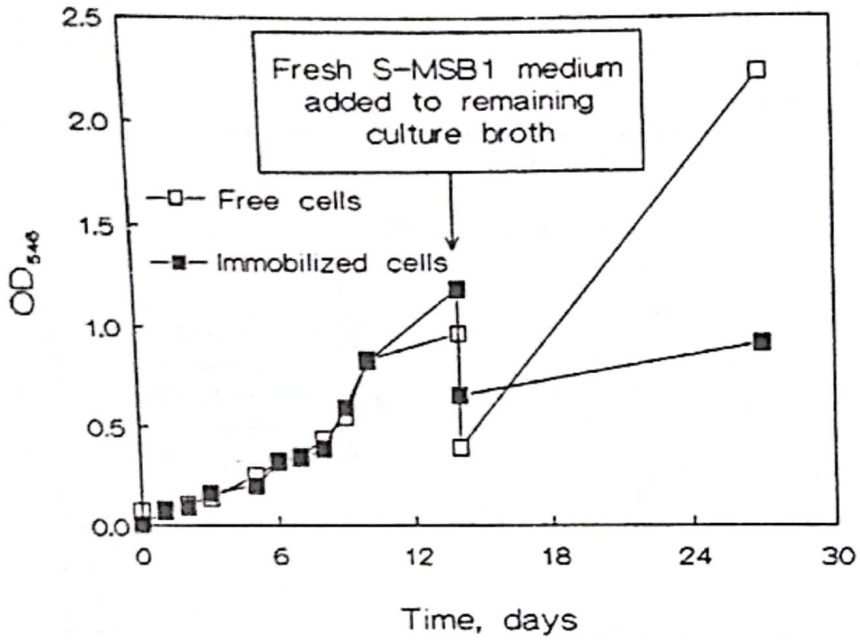

Figure (2): Optical density (OD_{546}) profiles of free and immobilized cells of Ar. globiformis SBI-5 cultures using polyurethane-coated coupons.

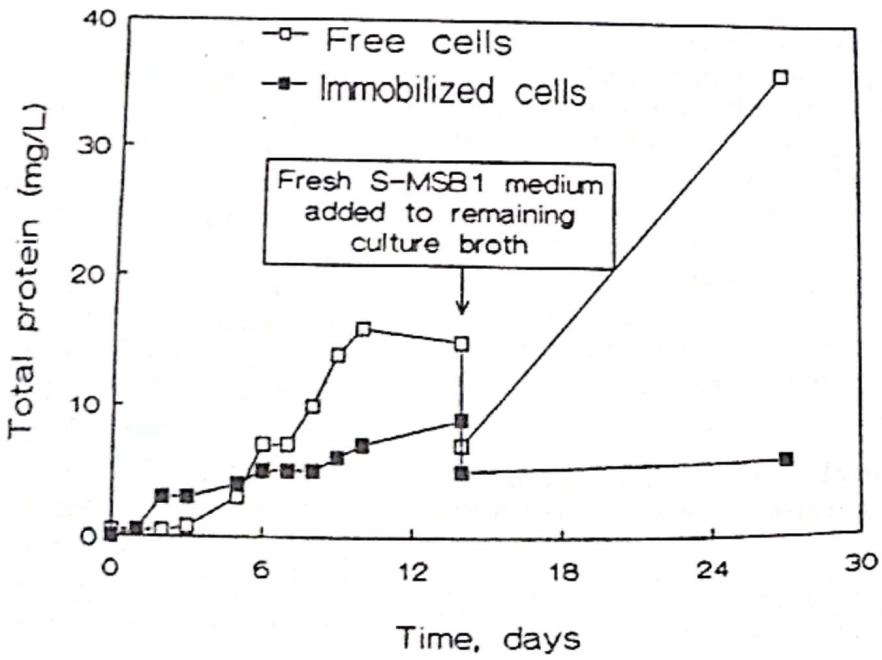

Figure (3): Total protein (mg/L) profiles of free and immobilized cells of Ar. globiformis SBI-5 cultures using polyurethane-coated coupons.

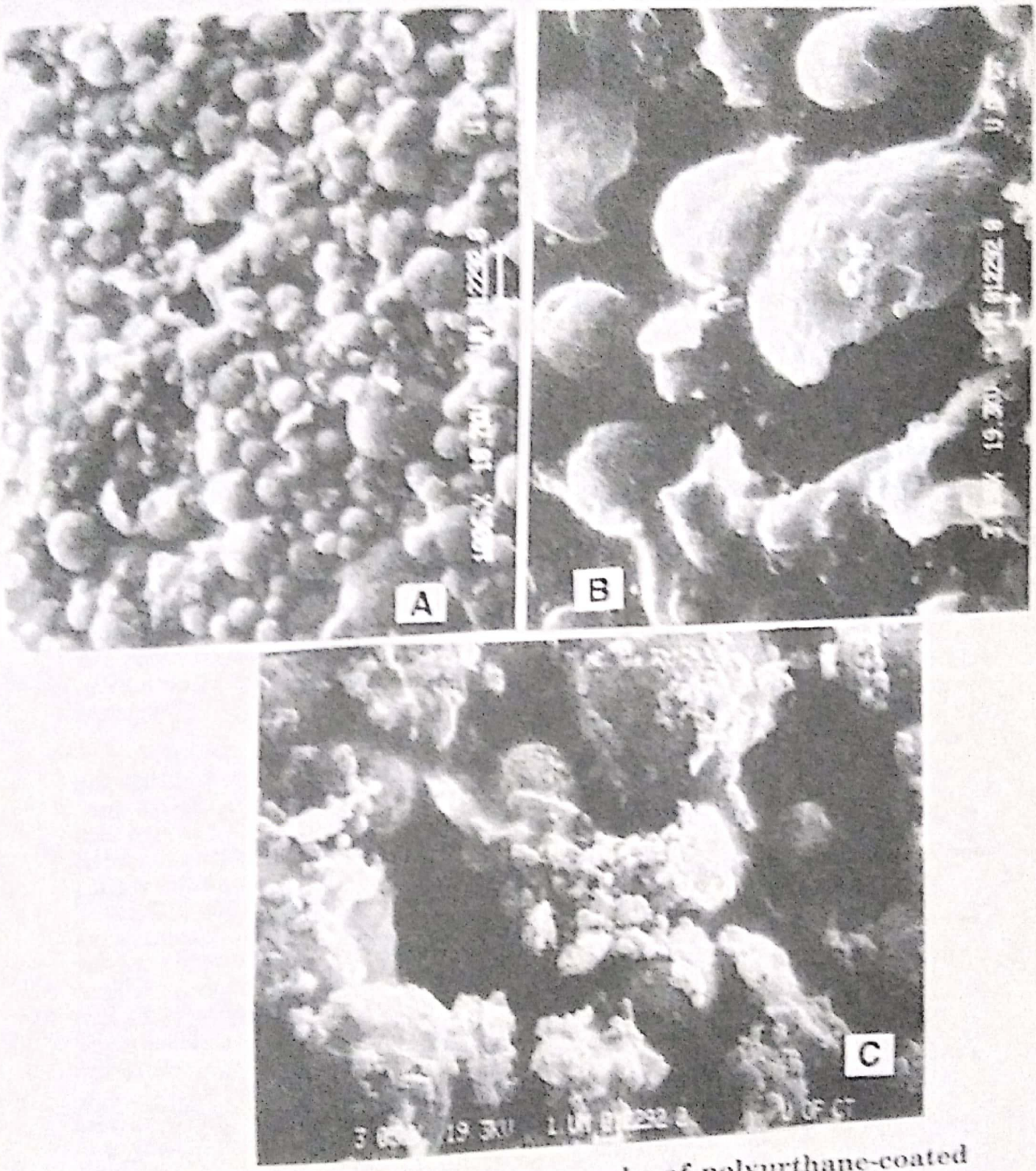

Figure (4): Scanning electron micrographs of polyurthane-coated coupons. (A) Untreated coupons (bar = 10 μm). (b) After fermentation with free cells of Ar. globiformis SBI-5 for 30 days (bar = 1 μm). (C) After fermentation with immobilized cells of Ar. globiformis SBI-5 for 30 days (bar = 1 μm).

free-cells began growing again at the end of 27th day, while the optical density and total protein of the culture broth were increased nearly five-fold. The addition of fresh S-MSB-1 medium appears to have had little effect, or perhaps impeded the growth of the immobilized cells. Reasons for such behaviour are not clear.

Both free and immobilized culture broths began to have a gray color within 192 hours, as pigment was released from the polyurethane coatings into the broth. The gray pigment material in the culture broths was not soluble and it was settled to the bottom of the tubes shortly after collection.

Visual examination of the immobilized cell culture show dark discoloration of the culture broth and similar discoloration on the surface of the polyurethane-coated coupon which was coated with calcium alginate and SBI-5 cells. Coupons in the SBI-5 culture containing free-cells retained their original gray color with no discoloration of their painted surfaces.

Scanning elctron micrographs of polyurethane-painted coupons recovered from the experiments are shown in Figures 4 (A to C). The surface of an uncultured polyurethane-painted coupon displays a torturous landscape composed of hillets 5-15 µm in diameter, valleys, and an occasional deep pit as shown in Figure 4A. Figure 4B shows the surface of a coupon taken from a culture containing free SBI-5 cells. Small groups and even single cells can be seen randomly dispersed over the hillet, rugose, wrinkled surface of the coupon. Note also that the rugosity of the hillet's surface is markedly reduced.

Figure 4C, shows heavy concentrations of cells and the thick, fibrous matting on the alginate-coated, polyurethane-painted coupon surface. The rugosity of the hillets is also markedly reduced. There is no discernable difference in the surface texture of coupons exposed to either immobilized or free-cells. Thus, surface texture by itself reveals no distinct biodegradative advantage in maintaining cells in close contact with the polyurethane surface of the coupons. However, observation only of surface texture does not reliably indicate to what depth and degradative process may have penetrated, or what total mass fraction of the film might have been removed. No yellow primer coating or bare metal surfaces were uncovered on any of the aluminum coupons tested during the biodegradative experiments. It appears that the biodegradative attack on the urethane coating is uniformly dispersed over the entire surface of the coupon. It appears that SBI-5 cells possess unique properties for the degradation of polyurethane. This work could lead to safer processes for stripping polyurethane from metal surfaces and for degrading polyurethane wastes collected by stripping polyurethane with chlorinated solvents.

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التحلل والتجريد البيولوجى لطلاء البولى يوريثان بواسطة البكتريا المحللة للهيدروكربونات

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تم زراعة خمسة عزلات محلية (SBI 1-5) على مستنبت الآجار المحتوى على املاح المعادن فى وجود طلاء البولى يوريثان كمصدر وحيد للطاقة والكربون . ونتج عن ذلك نمو جميع العزلات ما عدا العزلة رقم ٤ (SBI-4). كما تبين أن جميع العزلات لها القدرة على إنتاج خميرة الإستراز فى الوسط المكون من محلول الأملاح أو فى وسط النمو الغنى فى وجود رقائق الألمونيوم المطليه بالبولى يوريثان كما تم زراعة العزلة SBI-5 على وسط محلول أملاح المعادن فى وجود شرائح الألومنيوم المطليه بالبولى يوريثان كمصدر وحيد للطاقة والكربون حيث تم الاستدلال على التجريد البيولوجى لطبقة البولى يوريثان عن طريق ملاحظة تغير لون المستنبت والزياده فى الكمية الكلية للبروتين وزيادة الكثافة الضوئية لمزرعة الميكروب.

فى هذه الدراسة تم تثبيط عزله الأرثروباكتر جلوبيفورمز (SBI-5) على شرائح الألمونيوم المطليه بالبولى يوريثان بمساعدة طبقة رقيقة من اللجينات الكالسيوم حيث أدى ذلك إلى زيادة عتامة الوسط المحتوى على الخلايا المثبطه مقارنه بتلك المحتوية على الخلايا الحره. بالفحص المجهرى لشرائح الألمونيوم المطليه بالبولى يوريثان بواسطة الميكروسكوب الاليكترونى تم الكشف عن نقص ملحوظ فى الأشكال الهضبية للسطح المدهون عند المعالجة الميكروبيولوجية.