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RAPID BIOCHEMICAL CHARACTERISATION OF POLYURETHANE DEGRADING FUNGI USING AMPEROMETRIC BIOSENSOR TECHNIQUE

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Biodegradation of polymers has become a subject of immense research activity due to the technological importance. The biochemical characterisation of five fungi isolated from polyurethane disposal site is presented in this paper. The concept of biosensing has been exploited for the assimilation characterisation, tolerance to various inhibitors and optimisation of growth media. The preliminary studies indicate that five of these isolated are promising for the biodegradation of polyurethanes. Two of these organisms were identified as Aspergillus niger, A. tamerii and three species were identified as Fusarium solani, Fusarium oxysporum and Fusarium sp (under further identification). The biochemical characterisation of these fungi were carried out employing the principles of biosensors and the resistance to heavy metals and antibiotics were evaluated. The data obtained is based on the respiratory rate of microorganisms and is more rapid and reliable. The data obtained are discussed with reference to the individual fungus.

Keywords: Biochemical characterisation, biosensor, amperometry, isolation and polyurethane

INTRODUCTION

Polyurethanes are versatile class of polymers employed in diverse areas such as, agriculture, industry, commerce, and medicine, in an extensive range of materials such as foams, elastomers, paints, fibers, fabric coatings, adhesives and sealants [1]. The market for polyurethanes has been reported as increasing by an average of 10% per year, since 1955 [2]. Although it has been widely quoted to be a xenobiotic, polyurethane has been found to be susceptible to biodegradation by naturally occurring microorganisms [3] and the mechanism of degradation appears to involve microbial enzymes [4-6,1]. It has been shown that the polyether polyurethanes were markedly less susceptible to fungal attack than the polyether polyurethane [7]. Some fungi have been reported to have the ability to tunnel into the polyester polyurethane formulations [8-10].

Fifteen isolates brought into pure culture from test pieces of foam or elastomer has been reported to bring about clearing of polycaprolactone agar [5]. Researchers have reported the isolation of PU degrading organisms from jet-fuel, of deteriogens of the polyester polyurethane foam baffles in fuel tanks [11,12] and subsequently from foams [13,14], elastomers [15] and a wide range of other materials including foam baffles in aircraft, print rollers, automotive steering gates, cable sheathings, shoe uppers & soles, foam packing, cattle ear tags, automatic milking equipment, foam cushioning, water-proof clothing and sailing dinghy covers [4,16]. Pathirana and Seal [4] isolated Gliocladium roseum during soil burial experiments and showed its ability to deteriorate polyurethanes in the presence of basic organic nutrients. Deterioration of polyester polyurethanes was enhanced or even initiated by the presence of small amount of organic nutrients, however the use of complete media tended to delay utilisation of the polyurethane [17,18].

A detailed investigation on the biochemical characteristics of the microorganisms is necessary in order to develop a sensor especially for increasing its stability and improving its selectivity. Research on biosensors has grown phenomenally in recent years. Eleven microorganisms were characterized by the biosensor technique by injection of 30 different substrates and substrate mixtures. The advantage of

sensitivity and quantitative registration of signals, which enables recording of very low signals, have been studied. Many microbial sensors have been developed in the recent past, where the microorganisms are in direct contact with a transducer, converting the biochemical signal into quantifiable electrical response signal, which could be exploited for sensitive determination of a large spectrum of substances to monitor pollution.

TABLE I: Results of assimilation of substrates by organisms

Microorganism	Fusarium solani	Fusarium oxysporum	Fusarium sp.	A. niger	A. terreus
Substrates					
Amino acids					
DL Alanine	213.32	20.00	60.00	8.00	80.00
DL 2-amino n Butyric acid	213.32	5.00	---	---	---
L Arginine HCl	26.64	8.00	40.20	2.00	100.05
DL Aspartic acid	186.64	10.00	53.36	4.00	66.70
L Cysteine	---	5.00	26.68	---	33.35
L Glutamic acid	79.96	5.00	32.00	4.00	60.00
Glycine	106.64	10.00	24.00	---	60.00
Histidine HCl	53.32	5.00	16.00	4.00	60.00
DL iso leuceine	106.64	5.00	16.00	4.00	100.00
DL nor leuceine	79.99	5.00	8.00	2.00	60.00
L Leuceine	106.64	7.50	40.00	4.00	60.00
L Lysine HCl	26.64	2.50	12.00	4.00	40.00
DL methionine	79.96	5.00	10.00	2.00	100.00
L Ornithine HCl	---	5.00	20.00	2.00	240.00
DL B Phenyl alanine	373.32	6.00	30.00	---	360.00
L Proline	186.64	4.00	60.00	---	200.00
DL Serine	79.99	7.50	64.00	2.00	150.00
DL Threonine	106.66	6.67	24.00	6.00	150.00
DL Tryptophan	26.64	---	30.00	4.00	50.00
L Tyrosine	106.64	7.50	50.00	2.00	300.00
DL Valine	79.96	7.50	20.00	6.00	350.00
Organic acids					
Fumaric acid	133.39	8.00	24.00	4.00	---
Oxalic acid	---	---	---	---	
Citric acid	33.33	6.67	16.00	---	---
Acetic acid	333.33	20.01	20.00	25.00	260.00
Lactic acid	333.33	20.00	70.00	25.00	140.00
Maleic acid	---	5.00	---	5.00	---
Tartaric acid	26.66	20.00	---	6.00	---
Ascorbic acid	33.33	13.34	10.00	4.00	60.00
Formic acid	39.90	10.00	30.00	10.00	325.00

Table I Contd

Characterizations of microorganisms have been done using biosensor technique [19]. Based on the assimilation of the substrates, a rapid and continuous measurement of various compounds have been done [20]. Microbial sensors comprising immobilized whole cells and an oxygen probe were used for determination of substrates such as assimilable sugar [21], acetic acid [22], and alcohols in culture broth. Assimilation of organic compounds by microorganisms has been determined from the respiratory activity of the microorganisms, which could be directly measured by an oxygen electrode [22]. Microbial sensors have been developed for the determination of sugars, alcohols, organic acids, vitamins, antibiotics, peptides, enzyme activities and biochemical oxygen demand [23-25,21,26-30]. About fifty electrochemical sensors that utilize microbes have been developed for the determination of various organic compounds like sugars, alcohols, organic acids, vitamins, antibiotics, steroids, peptides, enzyme activities, and inorganic molecule (ammonia, nitrate, nitrite, sulfide, phosphate). Microbial electrodes monitoring the assimilation

TABLE I: Contd

Microorganism	Fusarium solani	Fusarium oxysporum	Fusarium sp.	A. niger	A. terreus
Alcohols					
Methyl alcohol	266.66	25.00	20.00	15.00	10.00
Ethyl alcohol	233.33	40.00	60.00	100.00	60.00
Propyl alcohol	399.98	20.00	60.00	5.00	10.00
Iso butyl alcohol	---	50.00	80.00	50.00	70.00
Iso amyl alcohol	---	50.00	40.00	35.00	130.00
Sorbiol	---	---	140.00	---	---
Mannitol	133.32	10.00	40.00	25.00	30.00
Glycerol	---	30.00	40.00	10.00	---
Carbohydrate					
Glucose	466.60	214.20	360.00	250.00	300.00
D Mannose	79.99	100.00	60.00	NA	50.00
D Maltose	6.66	20.00	30.00	40.00	50.00
Sucrose	126.60	150.00	40.00	25.00	100.00
Lactose	---	---	---	---	---
D Arabinose	53.32	50.00	4.00	20.00	25.00
Raffinose	39.99	---	4.00	25.00	40.00
D Fructose	26.66	50.00	15.00	40.00	60.00
L Arabinose	33.33	50.00	10.00	60.00	---
Trehalose	53.20	10.00	5.00	25.00	20.00
Cellobiose	26.66	15.00	25.00	25.00	20.00
D Galactose	33.33	50.00	25.00	25.00	25.00
D+ Melibiose	39.99	---	40.00	50.00	50.00
Dextrose	159.80	20.00	20.00	75.00	75.00

of substrates by measuring the respiration activity of the microorganisms have been developed for determination of assimilable sugars and other carbon sources like alcohols, organic acids and amino acids [21]. Various enzyme electrodes have been developed for the determination of sugars [31]. Enzyme sensors have been described for the determination of more than 80 different substances including cofactors, inhibitors and enzyme activities [31,32]. Biochemical oxygen demand (BOD) and ammonia in waste water could be also measured in the manner described above. Based on the decarboxylation of glutamic acid by glutamate decarboxylase, determination of glutamic acid has been determined by measuring carbon dioxide produced by the carbon dioxide gas sensing probe [21]. On line measurement of acetic acid in fermentation broth and glutamate sensor aided by microcomputer have been researched. Microbial electrodes consisting of immobilized whole cells and electrochemical devices such as oxygen electrode a fuel cell electrode or a combined glass have been developed and applied to the estimation of biochemical oxygen demand [33] the determination of antibiotics [34], and vitamins [35]. Assimilation characteristics of various microorganisms such as molds, yeasts, bacteria, actinomycetes and activated sludges were tested with various substrates. Key metabolites or rate of their metabolic overall reactions ATP [36], NADH, cytochrome content [37], and DNA, are suitable for describing the physiological state [38] have reported the simultaneous measurement of two parameters, the content of proteins and nucleic acids [39,40].

There have been reports that recognition of specific set of substances is responsible for the determination of complex variables like biodegradable compounds and mutagenic substances in waste water. Genetically engineered organisms can also be used as microorganisms for such sensors, especially for the determination of complex parameters such as BOD and mutagenicity [26,25,27,41]. Preliminary screening of mutagens has been done. In all the cases an oxygen electrode directly measures the assimilation activity of the microorganisms. It also has been found out that a very low microbial loading of the biosensor is a prerequisite for a kinetically controlled respiration electrode. It has been found out that for the sensors with very low 'microbe loading' and suitable immobilization of the microorganisms as well as thin membranes have to be used. The sensitivity of this type of sensor is mainly determined by the cell activity but not by diffusion limitation. It has been found out that one could attain response times of about 15 sec by very low 'microbe loading' of the sensor [19]. Selectivity of the sensor was improved by induction or blocking have desired or undesired transport systems. A combination of an electrode system with very low amounts of whole cells resulting in a decrease of diffusion resistance leading to very fast detection of changes in the respiration rates (1-5 sec) in contrast to microbial electrodes for assimilation test [21], having response times from 5 to 30 min has been reported [19].

This paper discusses the assimilation features of five fungi, isolated from soil where waste polyurethane scraps are thrown from an industrial site, manufacturing Polyurethane foams. The organisms have been tentatively identified as Aspergillus niger, Aspergillus tamerii, and three strains of Fusarium sp. The assimilation of Alcohols, Carbohydrates, Organic acids, Amino acids, and resistance to heavy metals and antibiotics has been studied.

EXPERIMENTAL

Materials

Potassium dihydrogen orthophosphate was purchased from Ranbaxy Laboratories, and all the other chemicals were purchased from Loba Chemie. All the analyses have been done using millipore water.

TABLE II: Resistance to heavy metals and antibiotics

Organisms	Fusarium solani	Fusarium oxysporum	Fusarium sp	A. niger	A. terrus
Heay metals					
Cadmium	30.00	---	30.00	11.30	30.00
Cobalt	10.00	40.00	60.00	20.00	80.00
Manganese	20.00	40.00	40.00	30.00	60.00
Strontium	220.00	40.00	40.00	30.00	13.00
Nickel	50.00	20.00	40.00	30.00	80.00
Zinc	---	---	---	---	30.00
Copper	30.00	50.00	50.00	50.00	40.00
Chromium	---	20.00	40.00	60.00	90.00
Iron	40.00	20.00	60.00	40.00	20.00
Mercury	20.00	30.00	20.00	10.00	10.00
Lead	---	---	90.00	40.00	20.00
Magnesium	50.00	60.00	---	---	---
Antibiotics					
Gryseofulvin	15.00	3.00	14.00	3.00	21.00
Mycoderm	12.00	8.00	19.00	6.00	16.00
Clotrimixozole	3.00	4.00	15.00	9.00	12.00
Ketagonazole	3.00	3.00	5.00	3.00	8.00

Isolation of microorganisms

A number of fungi were isolated from an industry in Vellore, Tamil Nadu, India manufacturing Polyurethane and five organisms were able to grow on the Polyurethanes as the sole carbon source. These have been tentatively identified as Aspergillus niger, A terrues, and three of Fusarium tamerii, Fusarium oxysporum and Fuasarium solani.

Culture of microorganisms

The fungi were cultured on 12 ml of the slant culture medium in the test tube of size 15 mm x 160 mm at 300 K for 2 days prior to assimilation tests. The fungal organisms were grown in Czapekdox media (KH_2PO_4 1 g, $NaNO_3$ 2 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01g, Sucrose 3.0 gms and Agar 15 gms in 1 litre of distilled water at *p*H 7.3).

Fabrication of oxygen electrode

A dissolved oxygen (DO) probe was fabricated using a gold cathode (area 0.03 cm^2), a platinum counter electrode and a Ag/AgCl reference electrode. The gas permeable membrane used in the probe was purchased from Century Instruments Co., Chandigarh. The biosensor, employed for the assimilation characterisation and other work was fabricated by coupling the immobilised membrane with the DO probe with the aid of dialysis tube and the details have been reported elsewhere. A Wenking Potentiostat Model POS 88 was used with a Rikadenki X-Y-t recorder.

Assembly of microbial electrode

Cellulose nitrate membrane (pore size < 0.25 micron) procured from millipore was used as the matrix for immobilisation. The microbe was immobilised by physisorption, after which the membrane was washed thoroughly with buffer to remove the loosely bound organisms on the membrane. A microbial dispersion (harvested during stationary growth phase) containing 0.2 gms of wet weight of the organisms/cm^3 was used for immobilization. The membrane retaining the microorganisms was placed on the teflon membrane of the oxygen electrode so that the microorganisms were trapped between the two membranes. The membranes were covered with nylon net and fastened with rubber rings. The immobilised membranes were stored at 227 K in phosphate buffer when not put to use.

Principle of assimilation and response of the microbial electrode

The initial current at zero time (i_0) was obtained with buffer solution saturated with DO, which indirectly shows us the endogenous respiration of the microbes. The assimilable substrate was injected into the system the substrate permeated through the porous acetyl cellulose membrane and

TABLE III: Comparison between the data by conventional technqiue obtained by studying the zone of inhibition and the data obtained by biosensor technique

Microorganism	Fusa 1		Fusa 2		Fusa 3		A. niger		A. Tamerii	
Substrate	C	S	C	S	C	S	C	S	C	S
DL Aspartic acid	+	++	+	+	+	+	+D	+-	+	-
Glycine	++	++	+	-	+	+	-	-	-	+
DL Isoleucine	+	++	-	+-	-	+-	+D	+-	+	+
Glucose	++	++	+	+	+	+	+	+	+	+
Surcose	-	+	-	+-	+D	+-	+D	+-	+	+
Formic acid	+	+	-	+-	+	+	-	+-	+	+
Ascorbic acid	-	+	-	+-	-	-	W	+-	-	+
Ethyl alcohol	++	++	-	+-	+	+	+	+	+	+
Methyl alcohol	+	+	-	+-	-	+-	+	+	+	+
TDA	D+	+-	-	X	+D	+-	-	+-	W	+-
Cadmium	D_	+-	D-	X	W	+-	+	+	+	++
Copper	D+	+-	W	+-	-	+-	++	++	+	++
Mercury	D+	+-	D+	+-	-D	+-	++	++	+	++
Gryscofulvin	-	+-	-	+-	+	+	+	++	+	++
Mycoderm	-	+-	-	+-	-	+-	+	++	-	+-

++ Very positive, + Positive, - Negative, D+ Delayed positive response

W Weak positive response, D- Delayednegative response, +- Variable response, X Data not available

was assimilated by the immobilised microorganism. The enhanced assimilation results in the increase in the respiratory activity of the microbe resulting in the decrease of DO at the microbial membrane/dissolved oxygen sensor interface. Hence, the current of the electrode decreased markedly with time until a steady state was reached since the respiration of the microorganisms was activated. The steady state indicated the diffusion of O_2 from a sample solution and O_2 consumption by microbes is in equilibrium. The steady state current depends on numerous factors among others such as concentration of substrate, the assimilation activity of the immobilised microorganisms and the rate of metabolism (oxidation) of substrate.

RESULTS AND DISCUSSION

All the five fungi were characterised by the amperometric sensor technique. The sensors with the five organisms were studied for 21 different amino acids, 14 different carbohydrates, 12 organic acids, 8 types of alcohols, and urea. 12 heavy metals and 4 antibiotics were studied for their inhibitory concentrations, by which we could find the resistance of the organisms.

Glucose was used as the standard substrate for all the organisms and Fusarium solani showed the highest response among the organisms studied. The assimilation capacity for glucose was found to be lower as seen from the Δi values recorded for Aspergillus niger. The others recorded good response for glucose. These results showed that the oxygen consumption varied among the microorganisms. Fusarium solani had excellent responses for all the aminoacids but could not assimilate Ornithine HCl even at concentrations of 5 ppm, except Fusarium sp. A. niger and A. tamerii were able to assimilate DL 2 amino n-butyric acid at concentrations of 5 ppm, 1 ppm and 5 ppm respectively. Aspergillus niger was not able to assimilate DL B phenyl alanine and L proline at concentrations of 2 ppm and 1 ppm. A. terreus showed good signals for all the aminoacids except DL 2 amino n butyric acid.

As expected none of the organisms was able to assimilate lactose but a very weak signal was obtained for Fusarium sp confirming the assimilation of lactose at concentration of 20 ppm. Even lactose as low concentration as 4 ppm was not assimilable by A. niger. Fusarium solani showed best responses to carbohydrates. Aspergillus niger, was not able to assimilate D^+ melibiose at low concentrations and weak responses were obtained for other organisms. Aspergillus terreus could not assimilate L. Arabinose.

Results of the substrate assimilation by microbial biosensor technique were compared with conventional data obtained by petriplate method. Although a large data were similar, there were diverged results which could be due to the slow rates of assimilation by the organisms, since biosensor technique is fast and registers changes in the oxygen consumption during a very short time.

Weak responses were obtained for citric acid for Fusarium solani, Fusarium oxysporum and Fusarium sp, and A. niger and A. terreus were not able to assimilate it. Fusarium oxysporum and A. niger were able to assimilate malic acid, but only weak signals were obtained by these two organisms confirming low levels of assimilation.

Fusarium solani showed good response for alcohols and the Δi value for ethyl alcohol was found to be maximum among the alcohols. A. terreus was not able to assimilate mannitol and others showed only a feeble response. Except Fusarium oxyporum, others showed weak responses to toluene diamine assimilation. None of the organisms was found to assimilate urea even at as low concentrations of 2 ppm.

A.niger and A. tamerii showed better resistance to heavy metals and antibiotics in relation to the other organisms studied. Fusarium solani, Fusarium sp. and A. terreus, had high resistance of 30 ppm for cadmium. A. terreus had a very high resistance of 80 ppm for cobalt. All the organisms showed a low resistance to mercury with maximum resistance shown by Fusarium oxysporum. Fusarium sp. had a high of 90 ppm as resistance for lead. Fusarium solani had high tolerance to mercury with 50 ppm as resistance concentration. A. terreus showed good resistance of 21 ppm for gryseofulvin and 16 ppm for mycoderm. A high resistance of 19 ppm of mycoderm and 15 ppm of clotrimixozole was recorded for Fusarium sp. A. terreus showed 8 ppm as maximum resistance limit for ketagonazole.

CONCLUSION

One of the advantages of this technique is the sensitivity besides the quantitative registration of very low signals. The data obtained could be used to compare different strains, mutant of transformants of one and the same species. Based on selective assimilation of the organisms, a potential organism for development of specific sensor could also be screened for further study.

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