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RESEARCH ARTICLE

FAST ASSIMILATION OF MIXED MICROBIAL (*Pichia nakasei, Hansenula anomala*) BOD SENSOR BY USING VARIOUS BIOLOGICAL SAMPLES

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ABSTRACT

Biosensors have been developed to estimate the BOD level of various pollutants. The main objective of this work is to develop a microbial biosensor which can be able to assimilate large number of biological samples and other substrates. The present study reveals that the yeast species *Pichia nakasei, Hansenula anomala* acted as a new alternate for BOD estimation. To develop microbial biosensor the above pure culture was mixed together in the ratio of 1:1 and immobilized on a special type of matrix and coupled under sandwich model. The constructed biosensor was connected with a BOD meter. The optimum temperature was determined and standardization was carried out using Glucose:Glutamic acid (GGA).Then the assimilation of different substrates (carbohydrate, amino acids, alcohols, organic acids, aldehydes and heavy metal ions) were analyzed. Finally BOD of different types of real samples was determined.

INTRODUCTION

The biochemical oxygen demand (BOD) is an index for biodegradable organic compounds in water and wastewater. This technique is widely used for the evaluation of water and wastewater quality (Eaton *et al.,* 1994). However, the conventional method for determining the BOD is time-consuming (5 days of incubation) and usually requires experience and skill to achieve reproducible results (Bourgeois *et al.,* 2001). Five-day BOD test method (BOD_5) has been widely used as the standard method to determine the concentration of biodegradable organics in wastewater (APHA, 1995). Moreover studies have been made to develop alternative methods based on the dissolved oxygen consumption (Karube *et al.,* 1977; Yang *et al.,* 1997) or photometric methods such as luminescence (Hyun et al., 1993) and fluorescence (Reynolds and Ahmad, 1997) for determining BOD. BOD sensors using microorganisms, such as *Pseudomonas putida* (Chee *et al.,* 1999), *Trichospwron cutaneum* (Murakami *et al.,* 1998), *S.marcescens* LSY4 (Kim and Kwon, 1999), *Arxula adeninivorans* LS3 (Tag *et al.,* 2000), were constructed. A mixture of microorganisms was developed for the purpose of

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on-line monitoring of BOD in the waste water. The general inert materials used to immobilize the living cells are nitrate cellulose membrane and acetate cellulose membrane. However, membrane-type BOD biosensors have several disadvantages: a great decline of DO resulting from the mass transfer resistance of the membrane; unstable biosensors and poor reproducibility of the measurement results because of the small amount of biomass immobilized in membrane; and high requirement for DO electrode. In most cases, intact microbial cells that contain active redox proteins are electrochemically inactive, as their cell walls and other surface structures are electrically nonconductive. Mediators can be used to facilitate the transfer of electrons from the microbial cells to the electrode (Park and Zeikus, 2000; Vega and Fernández, 1987). When a mediator is present in the reaction medium, it acts as an electron acceptor and is preferentially reduced during the metabolic oxidation of organic substances. The reduced form of the mediator is then reoxidized at a working electrode (anode), which is maintained at a sufficiently high electric potential. An Mediator less microbial Fuel Cell(MFC) system can be used for various purposes, including biosensors, bioelectrochemical synthetic processes, and electricity generation (Tayhas *et al.,* 1994). In particular, mediated MFCs have been studied as BOD sensors (Trosk *et al.,* 2001; Pasco *et al.,* 2004). *Pichia* (*Hansenula*) is a genus of teleomorphic yeasts in the family *Saccharomycetaceae*. The anamorphs of some *Pichia* species are *Candida* species. *Pichia* species are thought to be opportunistic pathogens (URL, 2006). One member of the genus, *P. pastoris*, is used as an expression system in molecular biology is *Hansenula anomala*.

MATERIALS AND METHODS

Chemicals

Glucose Glutamic Acid (GGA) was purchased from Sigma Aldrich Chemicals. Other reagents were commercially available analytical reagents of laboratory grade materials (Ranbaxy and Rankem).

Microorganism

The lyophilized pure cultures of *Pichia nakasei* (NCYC 1451) and *Hansenula anomala* (NCYC 1509) were used to construct the biosensor. Organisms were collected from Microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh. The growth medium consisted of $C_6H_{12}O_6$ 1g, peptone 0.5g, yeast extract 0.3 g, malt extract 0.3 g, agar 2 g and distilled water 100 ml. The pH of the medium was maintained at 7.0±0.2.

BOD Standard Solution

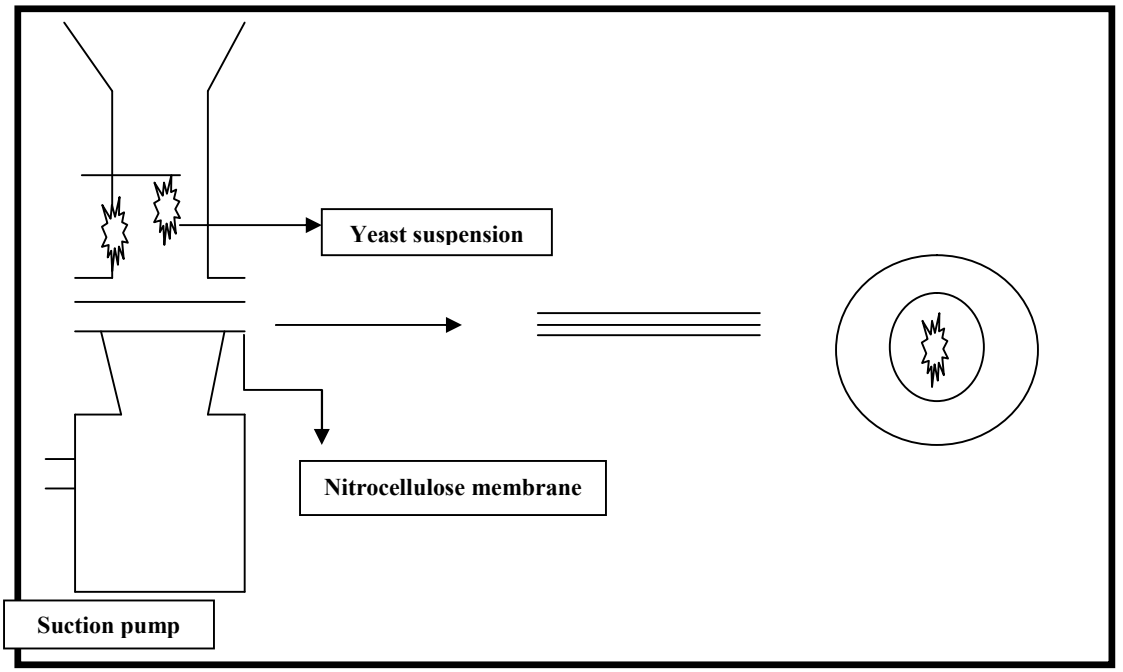
The BOD standard solution (150 mg/L glucose and 150 mg/L glutamic acid) was prepared according to standard procedures (APHA, 1995). This solution has a known BOD value of 198±31 mg BOD/L. Higher strength BOD standards were prepared by increasing the GGA loading and lower strength BOD standards were prepared by appropriate dilution with distilled water.

Immobilization

The *Pichia nakasei* and *Hansenula anomala* were adsorbed in the pore of nitrocellulose membrane by physisorption technique (Cass, 1990).The membrane was placed in a special holder and about 500 µl of the culture (yeast) was loaded, excess amount of buffer was filtered through the micromatt under suction pump process. After immobilization, the membrane was washed thoroughly with phosphate buffer to remove the loosely bound microbes on the micromatt. These membranes were involved in the microbial BOD sensor construction. Other immobilized membranes were stored at 4^oC in phosphate buffer. The BOD meter is used to carryout BOD measurement.

Schematic explanation of biosensor

The experimental apparatus consist of BOD meter. The immobilized membrane was coupled with dissolved oxygen membrane in sandwich model. Oxidation and reduction reactions were carried out at working electrode and current signals produced were monitored using a BOD meter. The biosensor was dipped in a phosphate buffer (pH 7.0). Magnetic stirrer was used for equal distribution of dissolved oxygen. When the steady state current was attained, synthetic BOD nutrient solution (GGA) 100 µl was injected into the biosensor along



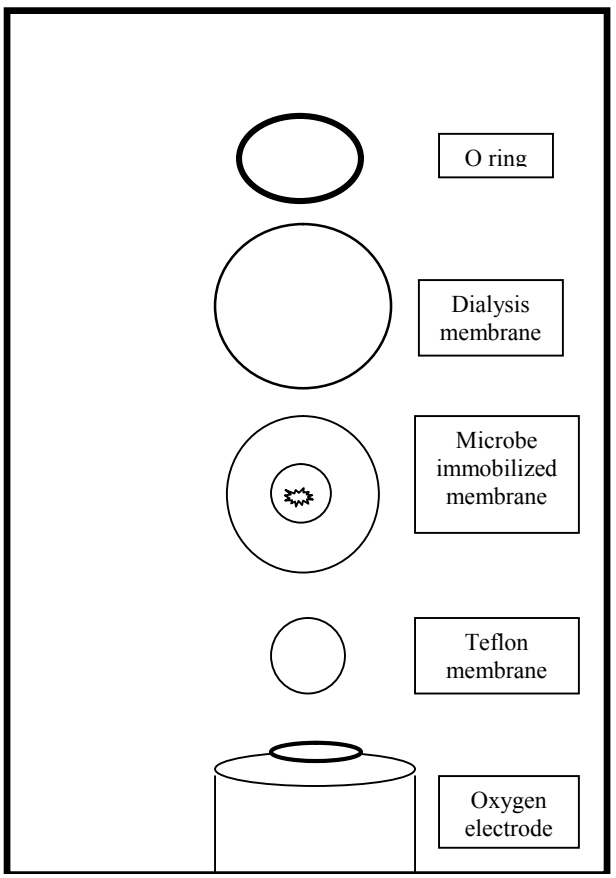


Fig. 1.Schematic diagram of biosensor

with the 5 ml of buffer, the reading was measured using a BOD meter for every 5 minutes. The current values of each addition calculated from the initial current which was known as steady state.

Effect of temperature

To detect the optimum temperature various temperature range were taken such as 5, 10,15,20,25,30,35,40 and 45 ^{o}C respectively.

Sample analysis

After standardization various organic substrate such as carbohydrates, alcohols, aldehydes, amino acids organic acids and heavy metal ions were used instead of GGA solution. Finally the sensor improved to assimilate for different sources and then the real samples were introduced to measure the BOD value.

RESULTS AND DISCUSSION

Effect of Temperature on Biosensor Response

The influence of temperature on the microbial activity and response of the biosensor was investigated. The biosensor gave its maximum response at a reactor temperature of 25^{o}C, as compared to that of other temperature ranges. Moreover, when the temperature was above 30^{o}C, the electrode response decreased slightly, which might be due to the inactivation of the microbial cells at higher temperature. The very lowest assimilation range was found at 5^{o}C (Fig. 2).

Standardization of GGA

The microbial sensor was standardized by Glucose Glutamicacid (GGA) solution. The assimilation response of *Pichia nakasei* and *Hansenula anomala* was found to be increased with increase in concentration of GGA (Fig.3).

Response of *Pichia nakasei* and *Hansenula anomala* for various substrates

The optimum temperature and assimilation of GGA were depicted in the fig 2&3.The optimum

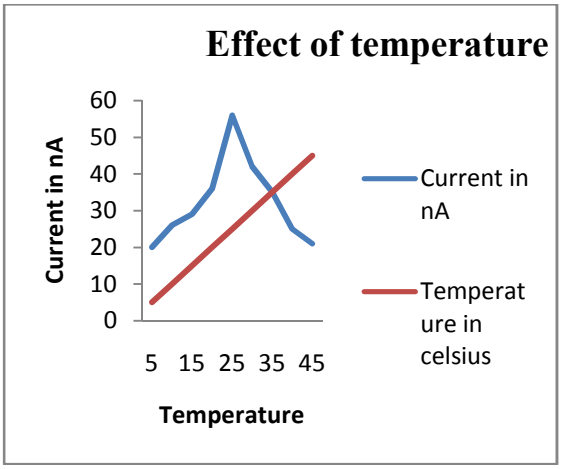


Fig. 2. Effect of temperature in current generation

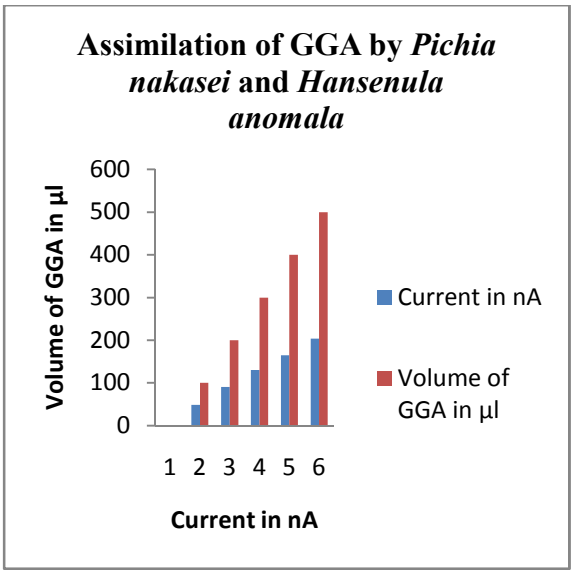


Fig. 3. Assimilation of GGA by *Pichia nakasei* and *Hansenula anomala*

temperature was found to be 25 ^{o}C and assimilation of GGA was increased in increasing concentration. The response of *Pichia nakasei* and *Hansenula anomala* assimilating various carbohydrates were found to be higher for glucose and lower for galactose. The results depict that, the sugar glucose is readily assimilated by the organisms. For amino acids the assimilation was found to be higher for tryptophan and lower for methionine. The response

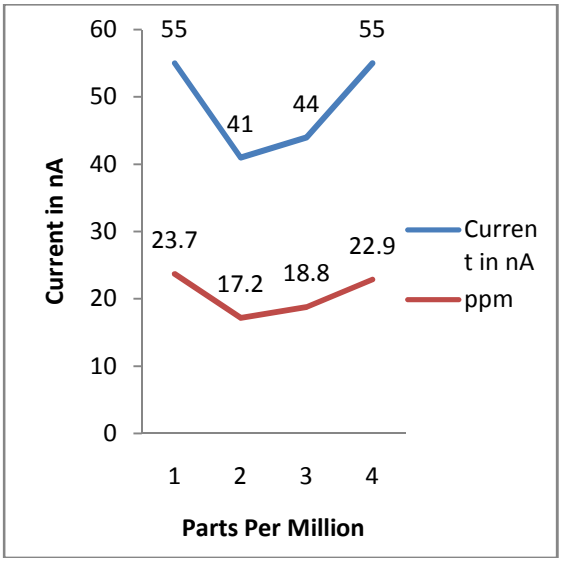


Fig.4. Response of *Pichia nakasei* and *Hansenula anomala* for various aldehydes

of *Pichia nakasei* and *Hansenula anomala* in assimilating various alcohols, organic acids, aldehydes and heavy metal ions were depicted in the table 1, 2, 3 & 4. For the real samples tested, better results were found in sugarcane juice followed by diary industry waste water and sago factory effluent (Table 5)

Table 1. Response of *Pichia nakasei* and *Hansenula anomala* for various carbohydrate samples

S.No	Carbohydrate	Current in nA	ppm
1	Glucose	65	51.0
2	Sucrose	27	27.4
3	Mannose	25	26.8
4	Galactose	17	21.2
5	Raffinose	54	36.8
6	Arabinose	61	39.0
7	Maltose	50	36.1
8	Lactose	47	32.0

The investigation revealed that this is a new alternative method for BOD estimation using *Pichia nakasei* and *Hansenula anomala* organisms and this fact could be taken into consideration for the preparation of some other mixed culture biocatalyst for the fabrication of a universal BOD probe. The efficiency of *Pichia nakasei* and *Hansenula anomala* microbial based sensor for the

Table 2. Response of *Pichia nakasei* and *Hansenula anomala* for various amino acid samples

S.No	Amino acids	Current in nA	ppm
1	Alanine	69	50.1
2	Aspargine	59	44.0
3	Phenyl alnine	59	44.2
4	Methionine	30	21.2
5	Valine	68	49.2
6	Tryptophan	72	50.8
7	Glycine	58	37.7
8	Lysine	60	45.3
9	Leucine	55	34.6
10	Arginine	63	47.9
11	Threonine	59	44.8

Table 3. Response of *Pichia nakasei* and *Hansenula anomala* for various organic acid samples

S.No	Organic acids	Current in nA	ppm
1	Gluconic acid	41	20.1
2	Fumaric acid	49	20.9
3	Sulfanilic acid	40	20.0
4	Tartaric acid	33	16.1
5	Nicotinic acid	34	17.6
6	Oxalic acid	37	19.7
7	Maleic acid	35	19.2
8	Ascorbic acid	34	17.8
9	Succinic acid	51	21.3
10	Formic acid	87	26.6
11	Salicylic acid	38	19.9

Tble 4. Response of *Pichia nakasei* and *Hansenula anomala* for various heavy metal ions

S.No	Heavy metal	Current in nA	ppm
1	Fe^{2+}	30	14.3
2	Mg^{2+}	25	12.0
3	Hg^{2+}	32	14.5
4	Ni^{2+}	22	09.6

Table 5. Response of *Pichia nakasei* and *Hansenula anomala* in assimilating various real samples

S.No	Samples	Current in nA	ppm
1	Pond water	28	17.2
2	Sago factory effluent	47	18.0
3	Sugar cane juice	83	25.3
4	Lake water	23	16.7
5	Spring water	16	15.1
6	Pharmaceutical effluent	13	6.6
7	Chemical industry effluent	24	16.9
8	Dairy industry waste water	50	24.3

assimilation of various organic substrates were more than equal to that of the usual biocatalysts *Trichosporon cutaneum, Bacillus subtilis* (Li *et al.,* 1994), *Torulopsis candida* (Sangeetha *et al.,* 1996). The probe was tested in batch mode. If it is tested under flow mode it can be directly used to the non-living monitoring. BOD sensor shows potential application for rapid estimation of biodegradable organic matter in waste waters. A short response time is the major advantage for using the BOD sensor system. Since it opens the possibility for on-line monitoring and process control (Siiri Velling *et al.,* 2005). MFCs using a single organism have an intrinsic disadvantage due to the limited range of fuel utilization. As such, electrochemically active microbial communities with different nutritional characteristics have been successfully enriched using fuel cell-type electrochemical cells (see below). Anode mediators were not used in these cases, and the coulomb yield was over 90% in some of the MFCs Similarly, an electrode placed in marine sediment can collect electrons through microbial reactions when connected to another electrode placed at the aerobic surface. Natural redox compounds, such as sulfur/sulfide, Fe(III)/Fe(II), and humic acid have also been suggested as possible mediators facilitating electron transfer from the microbial cells to the electrode (Bond *et al.,* 2002; Reimers *et al.,* 2001; Tender *et al.,* 2002).

Conclusion

In conclusion, this BOD biosensor can be used to measure the BOD value of the standard solution and the real samples. The BOD biosensor showed good reproducibility in the measure process and calibration procedure, which gave a response within 15 minutes. The optimum response of the sensor can be obtained at pH 7.0 and 30^{o}C. The sensor response is fairly constant over a period of 30 days, with about ±5% fluctuations. A short response time is the major advantage of using the BOD biosensor and it is applicable for rapid detection of BOD in water and other samples.

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