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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

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Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

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RESEARCH ARTICLE OPEN ACCESS

COMPARISON OF FUNGAL LACCASE PRODUCTION ON DIFFERENT SOLID SUBSTRATES, IMMOBILIZATION AND ITS DECOLORIZATION POTENTIAL ON SYNTHETIC TEXTILE DYES

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ABSTRACT

Laccases are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one electron transfer resulting in the formation of reactive radicals. In the current study 2 laccase producing strains of Aspergillus sp., and Penicillium sp., isolated from natural sources were studied for their optimal production on 5 different solid substrates (wheat bran, rice bran, ground nut cake, coconut cake and sesame cake). Wheat bran was found to be the substrate in which both the fungal strains were able to produce an optimal quantity of laccase enzyme of up to 1.632 IU/ml and 2.0 IU/ml respectively. The enzyme was extracted from the solid substrate having 2.0 IU/ml of enzyme unit and was immobilized on sodium alginate. When this immobilized alginate was allowed to act on the synthetic textile dyes Re Red BSID, Re Yellow merl, Orange merl, Red m5B and indigo carmine a maximum of 92.16% of decolorization was observed for Re Yellow merl after 5 days. All the other dyes were reduced to more than 70 % using the immobilized beads after 5 days.

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KEY WORDS

Laccase enzyme; Azo dye decolorization; Immobilized enzymes; Solid state fermentation.

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[I] INTRODUCTION

Dyes and dyestuffs are widely used within the food, pharmaceutical, cosmetic, textile and leather industries. Over 100,000 commercially available dyes exist and more than $7x10^5$ tonnes of dyestuff are produced annually [1, 2]. The human health impact of dyes used in the textile industry, especially azo dyes and their degradation products, has caused concern for a number of years, with legislation controlling their use being developed in a variety of countries [3]. Increasingly, the environmental and subsequent health effects of dyes released in textile industry wastewater are becoming subject to scientific scrutiny. Wastewater from the textile industry is a complex mixture of many polluting substances ranging from organochlorine-based pesticides to heavy metals associated with dyes and the dyeing process [4].

Microbial decolorization processes offer a complete cleanup of pollutants in a natural way as it reduces the color components to carbon dioxide, ammonia and water by initiating cleavage of the bonds in the dyes rather than creating possible toxic fragments of dyes [5]. Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one electron transfer resulting in the formation of reactive radicals [6]. The enzyme has been reported for several dye decolorizations [7, 8].

It was evaluated that laccase played an important role in the decolorization of wide spectrum dyes having diverse chemical structure, which suggested its implications in treating textile effluents as a low-cost and environmentally friendly technology [9, 10]. Nevertheless, laccase is often easily inactivated in waste treatment for the wide variety of treating conditions and is also difficult to be separated from the residual reaction system for reuse, which limits the further industrial applications of laccase. Enzyme immobilization technology is an effective means to make laccase reusable and to improve its stability [11], which is considered as a promising method for the effective decolorization of textile effluents.

The current study is focused mainly in the isolation of Laccase producing fungal strains from various sources. The isolated fungus will then be used in the production of Laccase enzyme on different solid substrates. The activity of the produced enzyme will be characterized under different physical conditions. The crude enzyme and the immobilized crude enzyme would be used in the decolorization of synthetic textile dyes.

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[II] MATERIALS AND METHODS

2.1. Isolation and screening of Laccase producing fungal strain

Fungal cultures were isolated from natural sources (soil samples, fruting bodies). Soil sample and spore suspensions were serially diluted and cultured on potato dextrose agar medium (PDA) and incubated at 30ºC. Selection of laccase producing organisms was done on plates containing following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH-6) supplemented with 0.02% guaiacol.

The isolated fungal strains were inoculated into these plates and the plates were incubated at 30°C for 7 days. Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium [12].

2.2. Identification of the screened fungal strains

Based on the reddish brown zones, the efficient strains of fungi selected were identified based on the standard Microscopic tests by lacto phenol cotton blue staining.

2.3. Comparison of Laccase production on different Agro Wastes

About ten agar discs of the two fungal strains were inoculated on 150 gm of 5 different solid substrates after proper sterilization (wheat bran, rice bran, ground nut cake, coconut cake and sesame cake) in a 250 ml Erlenmeyer flask. The flasks were incubated for a period of 10 days at 27^0C after which the enzyme component in the flask was estimated. The efficiency of the agricultural wastes on laccase production was compared statistically by correlation analysis.

2.4. Isolation of crude enzyme from the different solid substrates studied

The contents were extracted with sodium acetate buffer (pH 5.0, 10mm), filtered and centrifuged at 7000 rpm for 20 minutes at 4ºC. The supernatant was thus collected and used as enzyme source for the quantification of laccase enzyme.

2.5. Immobilization of crude enzyme with Sodium alginate

Ten milliliters of crude enzyme extract (Total laccase activity 2 IU) was added with sodium alginate solution (4%w/v) and properly mixed for about 20 minutes. It was added drop-by-drop by means of a Pasteur pipette into 50 ml of 100 mM calcium chloride solution; corresponding metal alginate beads were formed. After 45 minutes of hardening in each solution, the beads (about 3-4 mm in diameter) were separated from the hardening solution by filtration. All immobilized crude laccase alginate enzymes were kept in distilled water at 4ºC [13].

2.6. Characterization of immobilized laccase enzyme

2.6.1. pH stability

The effect of pH on enzyme immobilized beads and crude enzyme were compared and studied by incubating the samples in 0.1 M citrate-phosphate buffers (ph 2.0-6.0) for 24 hrs. Enzyme activity was estimated at the end of 24 hrs for all the different pH ranges as described in 2.9.

2.6.2. Thermostability

Thermal stability was assayed by incubating the enzyme immobilized beads and crude enzyme simultaneously at 60ºC for 240 minutes. At the end of 240 minutes the enzyme activity was measured at 30ºC as described in 2.9.

2.6.3. Storage stability

Immobilized beads were stored in 0.1M sodium acetate buffer (pH – 4.5) at 4ºC for several days. The enzyme activity was measured at 30ºC.

2.7. Decolorization studies of immobilized enzyme on different synthetic textile dyes

Decolorization potential of the immobilized enzyme (2.0 IU/ml) on 5 dyes (Re Red BSID, Re Yellow merl, Orange merl, Red m5B, Indigo carmine) were carried out on flasks containing 10mg/100ml of each dye on distilled water [13]. At the end of every 24 hrs the decolorization of each dye was measured at their absorption maxima. The percentage of decolorization was calculated by the formula

$$D=100(A\ i-A\ t)\ /\ A\ i$$

Where D was the Decolorization of the dye (in %), Ai the initial Absorption of the dye at its absorption maxima and At was the final absorption of the dye. The patterns of decolorization of the various dyes in relation to the time in days in relation to the immobilized laccase enzyme were analyzed using ANOVA.

2.8. Microtoxicity assay

Test cultures (Staphyloccus aureus, E. coli, and Trichoderma sp.) were cultivated on nutrient agar and potato dextrose agar plates for 24 hrs by spread plate technique. Two wells were bored in which the same concentrations (10 µl) of degraded and undegraded dyes were loaded. The plates were incubated for 24 hrs to 48 hrs to observe the zone of clearance around the wells bored [14].

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2.9. Extra-cellular Laccase activity assay

Enzyme activity was assayed at 30ºC by using 10mm guaiacol in 10mm acetate buffer containing 10% (v/v) acetone. The changes in absorbance of the reaction mixture containing guaiacol was monitored at 470nm (ε = 2.6 × 104M–1 cm–1) for 5 min of incubation. One unit enzyme activity was defined as the amount of enzyme that oxidizes 1µ mole of guaiacol per minute at 30ºC. The activities were expressed in IU/ml [15].

Table: 1. Enzyme production on different solid substrates

Fungal strains	Agro wastes used	Laccase activity (IU)
***Aspergillus* sp.**	Wheat Bran	1.632
	Rice bran	1.072
	Groundnut cake	0.973
	Coconut cake	1.433
	Seasame cake	1.267
***Penicillium* sp.**	Wheat Bran	2.0
	Rice bran	1.335
	Groundnut cake	1.299
	Coconut cake	1.351
	Seasame cake	1.063

[III] RESULT AND DISCUSSION

3.1. Isolation and screening of Laccase producing fungal strain

About 35 different fungus were isolated from natural sources and only 2 strains were found to be Laccase positive strains forming a reddish brown zone around the colonies inoculated.

3.2. Identification of the screened fungal strains

The two efficient fungal strains were identified as Aspergillus sp. and Penicillium sp. based on their microscopic observation. The first strain showed a septate and dichotomous hyphae, at 45o angle branching. Conidial heads are radiate to loosely columnar. Conidiophores are coarsely roughened, uncolored, vesicles spherical, metulae covering nearly the entire vesicle in biseriate species. Conidial heads radiate, uni- and biseriate; however, some isolates may remain uniseriate, producing only phialides covering the vesicle which are the characteristic features of Aspergillus sp. [16]. For the second fungus the hyphae were terverticillate and the conidia were spherical to elliptical in shape. Conidia were smooth and had a green color reflection in the mass. These microscopic features were found to be that of Penicillium sp. [17].

3.3. Comparison of Laccase production on different agro wastes

Wheat bran was found to be the effective substrate inducing a maximum enzyme production on both the isolated fungus. Aspergillus sp. produced up to 1.632 IU/ml of enzyme whereas Penicillium sp. produced the maximum of 2.0 IU/ml of enzyme after 10 days of incubation. On comparison rice bran and coconut cake were also found to be effective next to wheat bran. The Laccase enzyme production of both the organisms on different solid substrates was given in **Table–1**. Enzyme produced by Penicillium sp. (Maximum) was used in the immobilization and decolorization experiments. Similarly enhanced laccase production (>2 IU/ml) by S. psammoticus, was estimated when grown on wheat bran [18]. The value of the correlation coefficient for the laccase enzyme produced through the two fungus used was found to be + 0.67 (r value) which implies that the there is a positive correlation between the agricultural source and the enzyme produced i.e., any change in the feed used has an equal effect on the enzyme production.

3.4. Characterization of immobilized laccase enzyme

3.4.1. pH stability

The immobilized laccase was stable in the pH range 5–7 with over 80% residual activity, while the free laccase was stable in the pH range 4–5 **[Figure–1]**. This indicated that the immobilization appreciably improved the stability of laccase over a wide pH range. Similar results were observed for Adinarayana Kunamneni et al., 2008 [19] where the immobilized laccase enzyme exhibited a more stability over a wide pH range than that of the crude free laccase enzyme.

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3.4.2. Thermostability

The residual activity of the immobilized laccase enzyme reduced with time when incubated at 60ºC for 240 minutes. A maximum activity which was observed initially reduced from 100 % to 80% in the first hour of incubation. The enzyme activity was reduced continuously over the period of incubation. The residual activity at the end of 240 minutes of incubation was found to be about 37 % on compared to the 3 % of the crude extract **[Figure–2]**.

For Adinarayana Kunamneni et al., 2008 [19] where the immobilized laccase enzyme retained their activity over a longer period of time than that of the crude free laccase enzyme. Similar results were observed for Wang ping et al., 2008 [20] where the Thermostability of the immobilized enzyme was higher than that of the crude free laccase enzyme. The range of the immobilized laccase enzyme was over 80% even after incubating the immobilized enzyme at 60ºC for 60 minutes.

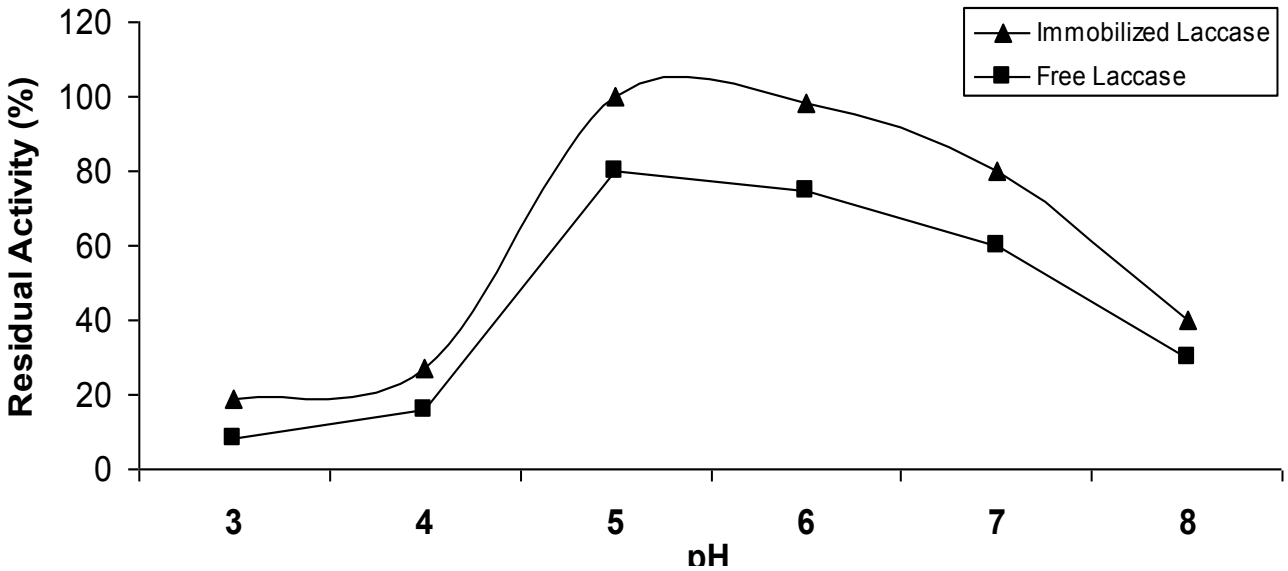

Fig: 1. Effect of pH on immobilized and free Laccase enzyme activity

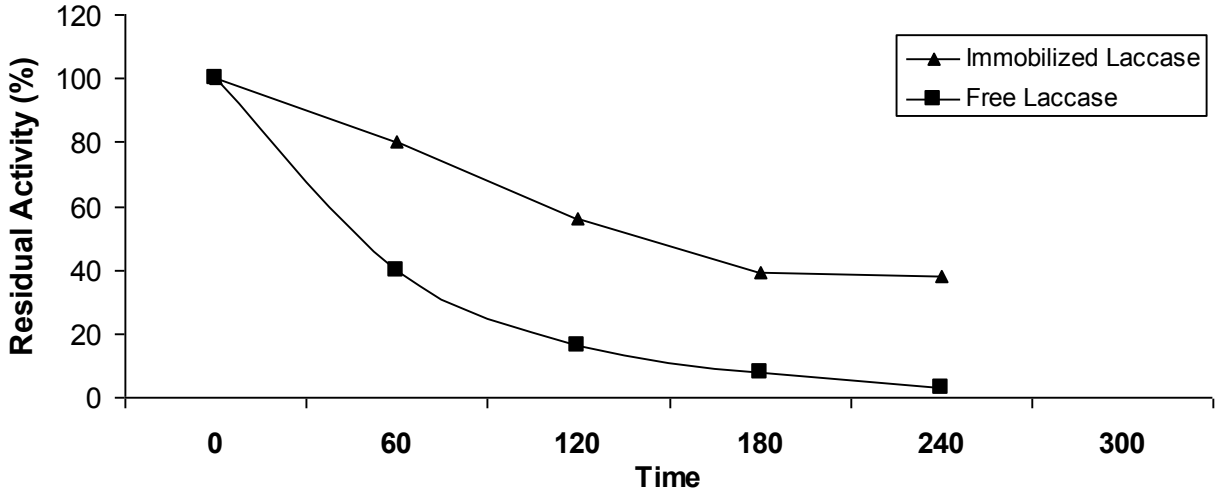

Fig: 2. Thermostability of immobilized and free Laccase

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3.4.3. Storage stability

During the first 2 months storage period there was a 2.5% drop in activity. Thereafter, the activity declined more slowly loosing an additional 1.3% in 2 months, equivalent to an approximate loss of 0.03% per day over the latter period and to a total loss of 3.8% in 4 months. Under the same storage conditions, the recovered activity of the soluble laccase amounted to 95 and 91%after 2 and 4 months storage, respectively. Leonowicz et al., 1988 [21] reported an increase in storage stability of laccase from Trametes versicolor immobilized on glutaraldehyde-activated aminopropyl porous glass.

3.5. Decolorization studies of immobilized enzyme on different synthetic textile dyes

A maximum of 92.16% of reduction was observed for Re yellow merl dye after 5 days of incubation with the immobilized enzyme **[Table-2]**. However, there exists a significant difference between the reduction percentage of the dyes upon the days of incubation (ANOVA, $p>0.05$). For all the dyes the percentage of reduction increased from the first day onwards till the 5th day. All the dyes under study were reduced by more than 80% with indigo carmine showing maximum resistance showing a reduction of up to 72.63% **[Table-2]**. There is no significant difference between the reduction percentage of the different dyes used (ANOVA, $p<0.05$).

RBBR decolourization by the immobilized copper-alginate enzymes, a rapid dye removal about 90% at 60 min [13] methyl red decolourization by the immobilized zinc-alginate enzymes, slightly increased up to 94% after 150min of the treatment [22]. In case of Indigo carmine decolourization was about 96% after 150min treatment with sodium-alginated immobilized enzyme. Bromophenol blue decolourization by immobilized calcium-alginate was about 84% within 150 minutes. The purified laccase could efficiently decolourize the indigo dye upto 80% within 4-5 days of incubation [13].

3.6. Microtoxicity assay

Microbial toxicity study on all the synthetic textile dyes showed the formation of zone and proved to be toxic to test organisms used (Staphylococcus aureus, E. coli, Aspergillus sp. Trichoderma sp. and Penicillium sp.) on compared to that of the degraded dye intermediates **[Table– 3]**. Among the dyes studied the azo dyes (Re Red BSID, Re Yellow merl, Orange merl and Red M5B) were found to be more toxic than the vat dye indigo carmine. This proves that the toxicity of the dye intermediates have reduced significantly on comparison with that of the dyes used in the study.

Table: 2. Reduction percentage of the synthetic dyes by immobilized Laccase enzyme

Dye used	Days of Incubation				
	Day 1	Day 2	Day 3	Day 4	Day 5
Re Red BSID	25.84	38.69	59.89	75.6	89.26
Re yellow merl	22.15	37.15	63.26	81.5	92.16
Orange merl	37.63	38.59	56.43	78.19	84.26
Red M5B	30.12	44.15	58.72	77.26	85.32
Indigo carmine	39.26	45	51.14	63.47	72.63

Table: 3. Microtoxicity studies of the synthetic dyes against standard test organisms

Test Organisms	Re Red BSID		Re yellow merl		Orange merl		Red M5B		Indigo carmine	
	S	T	S	T	S	T	S	T	S	T
S. aureus	+	-	+	-	-	-	+	-	-	-
E. coli	-	-	+	-	+	-	-	-	+	-
Trichoderma sp.	+	-	-	-	+	-	+	-	-	-

S – Synthetic dyes; T-Treated synthetic dyes; + Zone of clearance; - No zone of clearance

According to the work of Mane et al., 2008 [14] the dye Navy blue RX was toxic for the growth of agriculturally important microorganisms Azotobacterium sp and Pseudomonas aeruginosa. This made a conculsion that the dyes metabolites are not only non-toxic but stimulatory to agricultural crops and microorganism.

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[IV] CONCLUSION

From the current study it was concluded that immobilized laccase enzyme could potentially be used as a replacement for the conventional dye decolorization using microorganism. The main advantage in the immobilized enzyme treatment was that the time required by the organism to produce the enzyme was neglected and the decolorization rate will be faster in terms of the number of days or hours that the dyes need to be treated.

CONFLICT OF INTERESTS

I do hereby inform you that there is no conflict of interest with regard to the financial commitments, location at which the work done and with the number and order of co-authors in publishing this article in your esteemed journal.

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CASE REPORT OPEN ACCESS

CALCIUM ALGINATE AS A SUPPORT MATERIAL FOR IMMOBILIZATION OF L-AMINO ACID OXIDASE ISOLATED FROM ASPERGILLUS FUMIGATUS

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ABSTRACT

Crude L-amino acid oxidase (L-aao) of Aspergillus fumigatus was immobilized in various solid supports namely, entrapment in calcium alginate gel and gelatin gel, and adsorption in nylon membrane by cross linking with glutaraldehyde. Immobilization of A. fumigatus L-aao in calcium alginate gel was the most favourable since the percent entrapped activity was maximal in calcium alginate beads (31.77%) as compared to the adsorption in nylon membrane (26.88%). Maximum enzyme activity was found with 3 % sodium alginate. Reaction time for immobilized beads increases from 2h reaching a maximum at 6h. The activity of the calcium alginate entrapped A. fumigatus L-aao beads was assayed for four cycles with DL-alanine as the substrate. The enzyme showed 87.52 % activity during the second reuse and 72.37 % activity on its third use. Immobilization in gelatin gel was ineffective since the immobilized gel cubes were unstable and disintegrated during the enzyme reaction. As for immobilization of the L-aao by adsorption in nylon membrane, only 26.88% activity was retained.

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KEY WORDS

L-amino acid oxidase; Aspergillus fumigatus; immobilization; calcium alginate.

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[I] INTRODUCTION

Immobilization of enzymes is a technique, adopted to reduce the cost and increase the utilization of enzymes. Immobilized enzymes are widely used in different industries, especially in food and pharmaceutical and offer several advantages over bulk or free enzymes. Advantages include high productivity, automation, continuous processing, precise control of the extent of reaction, easy product recovery and non-contamination of the final product by the enzyme [1].

The determination of amino acids is important in several applications, and it is often the determination of single amino acids which is desired. In the clinical laboratory, determination of certain amino acids in physiological fluids or tissue may be useful indicator of certain diseases or disorders. Besides numerous chromatographic and electrophoretic methods developed for the determination of amino acids, including also chiral determinations, a method for the efficient determination of amino acids of particular chiral configuration can be developed by employing the stereoselective L-aao as biosensors or enzyme electrodes [2].

Two basic factors, crucial to the success of construction of biosensors are the method of immobilization of the enzyme and the selection of the most suitable signal transducer. Enzymatic biosensors for amino acids have already been described in analytical and biochemical literature, differing as to the kind of enzyme used, the method of enzyme immobilization employed and the type of transducers utilized. Immobilized L-aao degrades L-amino acids to 2-oxo acid, ammonia and hydrogen peroxide [3]. Amperometric biosensors for the determination of D- and L-enantiomers of L-leucine were developed by physically immobilizing L-amino acid oxidase in diamond paste [4].

Aspergillus fumigatus L-aao is an important enzyme that causes the racemic resolution of DL-alanine to produce D-alanine [5]. In the present study, different methods of immobilization were investigated with *A. fumigatus* L-aao. These include entrapment in calcium alginate gel, entrapment in gelatin gel, and adsorption in nylon membrane by cross linking with glutaraldehyde. The efficiency of an immobilization process can be measured by the following criteria. Most important of all, a high percentage of the enzymes must be initially retained in the gel matrices. Secondly, the enzyme activity must be preserved. And thirdly, the enzymes must be physically restrained from diffusing back into the substrate solution at a later time. Studies were done looking into the above aspect.

[II] MATERIALS AND METHODS

2.1. Isolation of crude L-aao of A. fumigatus

A. fumigatus cells (96 h old) were harvested by centrifugation at 9400 g and 10°C, for 10 minutes. The cells were then homogenized in a French pressure cell press (Thermo spectronic, USA) at 1500 psi for 5 minutes in ice cold condition. The cell suspension obtained was centrifuged at 13600 g for 15 minutes at 4°C. The cell debris was discarded and the clear supernatant was taken as the crude enzyme.

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2.2. Entrapment in Calcium alginate beads

Crude L-aao (5mL or 7.2 mg) was mixed with 3% sodium alginate solution in 1: 2 ratio. The immobilized enzyme beads were formed by dripping the enzyme-alginate mixture from a height of approximately 20 cm, drop wise into the calcium chloride (0.2 M) solution with continuous shaking. The beads were washed 3-4 times with distilled water and finally with 50 mM sodium phosphate buffer, pH 7.2. The beads were dried and weighed for further studies.

2.2.1. L-amino acid oxidase activity of the calcium alginate beads

The beads (2 g) were added to 10 mL of 50 mM sodium phosphate buffer, pH 7.2 containing 50 mM of the substrate i.e. DL-alanine. The reaction was performed for 2-7 h at 30ºC, 200 rpm. The enzyme assay was performed with 0.5 mL of the reaction filtrate as described previously [5]. Briefly, 0.5 mL of the reaction mixture was diluted 5 times with distilled water and reacted with 0.4 mL of 2, 4-dinitrophenylhydrazine (0.2% saturated in 2N HCl) for 10 minutes. To this 1.5 mL of 3M NaOH was added and absorbance at 550 nm was recorded after 15 minutes.
One unit of L-aao activity was defined as the amount of enzyme producing 1µmol pyruvate/min under the standard assay conditions.

2.2.2. Effect of sodium alginate concentration

Various concentration of sodium alginate (1% - 4%) were used to acquire beads with greater stability. Sodium alginate solution (1- 4 %) were prepared. The rest of the procedure for enzyme immobilization was same as stated previously.

2.2.3. Reusability of the calcium alginate immobilized beads

The L-aao activity of the calcium alginate immobilized beads was assayed for four cycles with 50 mM DL-alanine as the substrate, in order to find out the reusability of the entrapped enzyme. The enzyme reaction was performed for 6h under the conditions described earlier.

2.3. Entrapment in gelatin gel

Crude L-aao (5mL or 7.2 mg) was mixed with the 10 % gelatin solution in 1: 2 ratio. 2mL of the hardening solution (20 % vol. formaldehyde, 50 % vol. ethanol and 30 % vol. water) was added to the enzyme-gelatin mixture. The solution was poured into a mold or a small beaker and frozen at 0ºC for 4 h to facilitate the gel formation. When the gel was set, it was cut into small cubes of approximately 3mm per side. The gel cubes were washed once with distilled water and finally with 50 mM sodium phosphate buffer, pH 7.2. The enzyme activity was checked for the gelatin beads.

2.4.Cross linkage in nylon membrane

To a mixture of the L-aao (100 µL) and BSA (166 µL of 40 mg/mL), 166 µL of 2.5 % glutaraldehyde was added to initiate cross linking. The mixture was layered onto a prewetted nylon membrane of pore size 0.22 µm and the solution was allowed to cross link at room temperature (20-25ºC) for 2-3 h until a yellowish hard gel layer is formed. The enzymic membrane was washed with 50 mM sodium phosphate buffer, pH 7.2 to remove excess glutaraldehyde and the L-aao activity was determined.

2.4.1. L-aao activity of the enzymic membrane

The enzymic membrane is immersed in 4 mL of 50 mM sodium phosphate buffer, pH 7.2 containing 50 mM of substrate i.e. DL-alanine. The reaction was performed for 3-5 h at 30ºC, 200 rpm. The enzyme assay was performed with 0.5 mL of the reaction filtrate as described previously and the enzyme activity was recorded.

2.4.2. Reusability of enzyme immobilized nylon membrane

The L-aao activity of the enzymic membrane was assayed for two cycles with 50 mM DL-alanine as the substrate, in order to find out the reusability of the entrapped enzyme. The enzyme reaction was performed as before.

2.5. Statistical analysis

The experiments were done in triplicate and the data are expressed as mean ± standard deviation.

[III] RESULTS

3.1. Effect of sodium alginate concentration on the stability of the calcium alginate beads

Various concentration of sodium alginate (1% - 4%) was used to acquire beads with greater stability. The maximum enzyme activity was found with 3 % sodium alginate. At lesser concentrations of sodium alginate i.e. 1% and 2%, the beads were not formed properly and were very unstable. Also, maximum leakage of the enzyme occurred owing to the large pore size of the less tightly cross linked fragile calcium alginate beads. When the sodium alginate concentration was increased to 4%, the enzyme activity was comparatively very low, which might be due to the high viscosity of the enzyme entrapped beads, which decreased the pore size and thus hindered the penetration of the substrate into the beads.

3.2. Calculations of the L-aao activity after entrapment in calcium alginate beads

The efficiency of an immobilization process can be measured by the criteria that high percentage of the enzyme must be initially retained in the gel matrices. Keeping this in mind, the percent entrapped activity of the immobilized enzyme was calculated. The enzyme activity was recorded as the Units/g bead.

Initial activity of the free enzyme = 0.03024 µmol/min/ml or Units/mL
Volume of enzyme solution taken for immobilization = 5 mL
Weight of beads formed after immobilization of enzyme solution = 14 g
Enzyme solution entrapped in 2 g beads = 0.714 mL
Therefore enzyme entrapped =0.02156 Units/ 2g beads (on the basis of free enzyme)
Activity of immobilized enzyme obtained in 2g beads = 0.00685 Units/ 2 g beads
Therefore enzyme activity after entrapment = 31.77 %

3.3. Effect of reaction time on the activity of the calcium alginate beads

The effect of the reaction time on the activity of the immobilized enzyme was investigated by performing the enzyme reaction for different time periods. The enzyme activity, recorded as the Units/g bead increases from 2h reaching the maximum at 6h and then decreases as the reaction time is increased to 7h **[Figure-1].**

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3.4. Reusability of the calcium alginate beads

The activity of the entrapped enzyme was assayed for four cycles with DL-alanine as the substrate. The enzyme showed 87.52 % activity during the second reuse and 72.37 % activity on its third use [Figure-2]. Loss of the enzyme activity of the entrapped enzyme was observed during the fourth cycle. This decrease in the enzyme activity was due to the leakage of the enzyme from the beads. Thus the loss in activity on the second reuse was only 27 %.

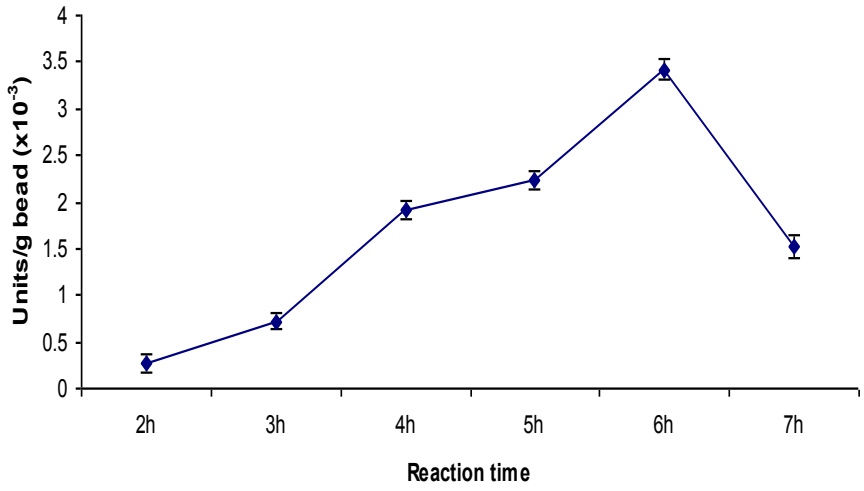

Fig: 1. Enzyme activity of the calcium alginate beads at different time periods. Reaction was performed from 2-7h under the standard conditions. Data are expressed as mean of three individual experiments ± standard deviation.

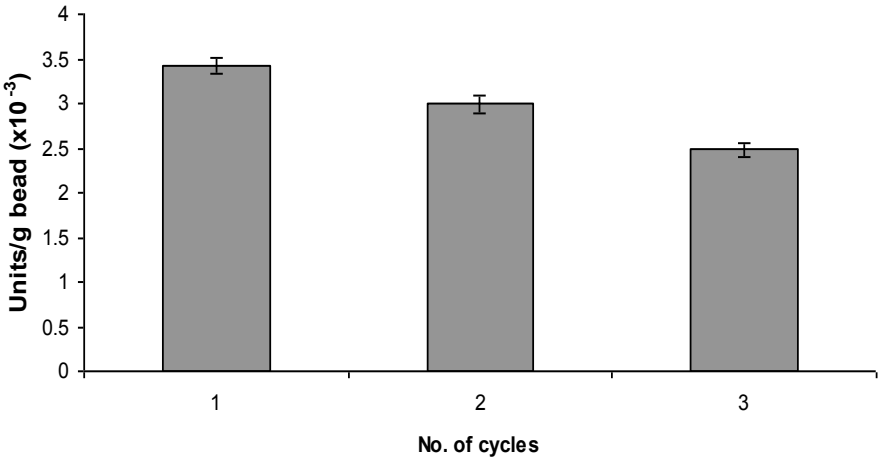

Fig: 2. Repeated use of immobilized L-aao beads showing the number of times the immobilized enzyme can be used. Data are expresses as mean of three individual experiments ± std. deviation.

3.5. Immobilization of L-aao of A.fumigatus in gelatin gel

The immobilization of L-aao in gelatin gel was ineffective since the immobilized gel cubes were unstable and disintegrated during the enzyme reaction. Thus, this method of immobilization is not feasible for the enzyme.

3.6. Immobilization of L-aao of A.fumigatus by cross linking in nylon membrane

3.6.1. L-aao activity of enzymic membrane

L-aao was immobilized in 0.22 µm nylon membrane by cross linking with 2.5 % glutaraldehyde. L-aao activity was assayed for the enzymic membrane and was recorded as the µmol of pyruvate formed /min / membrane or Units/ membrane.

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Calculation of enzyme activity:
Initial activity of free enzyme = 0.03024 Units/mL
Therefore, enzyme activity in 100 µL enzyme = 0.003024 Units/mL
Enzyme activity of the enzymic membrane = 8.13 x 10^{-4} Units / membrane (Reaction performed for 5 h)
Therefore, enzyme activity after cross linking = 26.88 %
Thus after cross linking in nylon membrane, only 26.88 % of the enzyme activity is obtained.

3.6.2. Reusability of the enzymic membrane

The L-aao activity of the membrane was assayed for two cycles in order to check the reusability of the cross linked enzyme. Complete loss of the enzyme activity was observed at the second use of the membrane. This may be due to leakage of the enzyme from the membrane during the enzyme reaction. This suggests that the cross linking of *A. fumigatus* L-aao by glutaraldehyde on nylon membrane is not suitable for reuse.

[IV] DISCUSSION

The determination of amino acids is important in several applications, and it is often the determination of single amino acids which is desired. Besides numerous chromatographic and electrophoretic methods developed for the determination of amino acids, including also chiral determinations, a method for the efficient determination of amino acids of particular chiral configuration can be developed by employing the stereoselective L-aao as biosensors or enzyme electrodes [2].

Two basic factors, crucial to the success of construction of biosensors are the method of immobilization of the enzyme and the selection of the most suitable signal transducer. Enzymatic biosensors for amino acids have already been described in analytical and biochemical literature, differing as to the kind of enzyme used, the method of enzyme immobilization employed and the type of transducers utilized. A specific multi enzyme biosensor for L-alanine has been developed with the use of alanine dehydrogenase combined with salicylate hydrolase and pyruvate oxidase on a Teflon membrane covering a Clark amperometric oxygen sensor [6].

The immobilization of *A. fumigatus* L-aao in calcium alginate gel was the most favorable since the percent entrapped activity was maximal in calcium alginate beads (31.77%) as compared to the adsorption in nylon membrane (26.88%). The entrapment of enzyme in calcium alginate is one of the important methods of immobilization. Alginates are commercially available as water soluble sodium alginates and they have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying and film forming agent. Entrapping within insoluble calcium alginate gel is recognized as a rapid, non-toxic, inexpensive and versatile method for immobilization of enzymes as well as cells [7].

The maximum enzyme activity was found with 3 % sodium alginate concentration. At lesser concentrations of sodium alginate i.e. 1% and 2%, the beads were not formed properly and were very unstable. Also, maximum leakage of the enzyme occurred owing to the large pore size of the less tightly cross linked fragile calcium alginate beads. When the sodium alginate concentration was increased to 4%, the enzyme activity was comparatively very low, which might be due to the high viscosity of the enzyme entrapped beads, which decreased the pore size and thus hindered the penetration of the substrate into the beads. Thus, 3 % sodium alginate concentration is most suitable for the immobilization of the *A.fumigatus* L-aao. This is in agreement with Farag and Hassan, 2004 [8] who report that the sodium alginate concentration ranging from 2-3 % was suitable for the immobilization of keratinase, lipase and protease.

The enzyme activity increases from 2h reaching the maximum at 6h; whereas, the unimmobilized or the free enzyme shows the maximum enzymatic activity at 1 h of reaction time. Thus the immobilized enzyme achieves the maximum enzyme activity at a reaction time 6 times higher as compared to the free enzyme. This increase in time is due to the time required by the substrate molecules to penetrate into the beads and reach the active sites of the enzyme. This observation is supported by Qader et al., 2007 [9], who reported that calcium alginate entrapped dextransucrase took 60 minutes to achieve the maximum enzyme activity, which was 4 times higher than the free enzyme.

Reusability of immobilized enzyme is a very important criterion for assaying its applicability in industry and is also a characteristic of immobilization. Immobilized L-aao in calcium alginate beads showed 87.52 % activity during the second reuse and 72.37 % activity on its third use. Loss of the enzyme activity of the entrapped enzyme was observed during the fourth cycle. This decrease in the enzyme activity was due to the leakage of the enzyme from the beads. The loss in activity on the second reuse was only 27 %. This observation coincides with the fact that alpha-amylase entrapped in calcium alginate beads was reused for 6 cycles with approximately 30 % loss in activity [10]. However, the immobilized *A.fumigatus* L-aao calcium alginate beads can be effectively used for 3 cycles.

[V] CONCLUSION

Thus, 3 % calcium alginate was the suitable support for immobilization of *A. fumigatus* L-aao since the percent entrapped activity was maximal in calcium alginate beads (31.77%) as compared to the adsorption in nylon membrane (26.88%).

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CONFLICT OF INTEREST

Authors declare no conflict of interest

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RESEARCH ARTICLE OPEN ACCESS

STUDY THE EFFECT OF CHLORPYRIFOS ON ACETYLCHOLINESTERASE AND HEMATOLOGICAL RESPONSE IN FRESHWATER FISH CHANNA PUNCTATUS (BLOCH)

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ABSTRACT

The toxic effect of chlorpyrifos (CPF) on biochemical and hematology in different concentrations and exposure periods was investigated in the fish, Channa punctatus (C. punctatus). The LC50 - 96h of technical-grade CPF was evaluated 811.98 µg/l for C. punctatus in a semi-static system and on the basis of LC50 value two sublethal concentrations viz. 203.0 and 68.0 µg/l were determined. C. punctatus were exposed to sublethal concentrations of CPF for 1, 3, 7, 14 and 21 days. The AChE activity, erythrocyte, hemoglobin percentage, haematocrit and leucocyte were decreased significantly ($p<0.01$) as concentrations and exposure periods increased. The highest reduction in AChE activity was recorded in brain followed by gill and blood plasma in both sublethal concentrations. Thus our results suggested that AChE activity and blood parameters could be used as potential biomarkers for environmental contaminants in aquatic system.

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KEY WORDS

Chlorpyrifos; Biochemical parameters; Hematology; Channa punctatus

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[I] INTRODUCTION

Chlorpyrifos [O, O-diethyl-O-(3, 5, 6-trichloro-2-pyridyl) phosphorothionate] is a broad spectrum organophosphate pesticide widely used in agriculture and residential pest control throughout the world. As a consequence of chlorpyrifos (CPF) widespread use in crop fields, it easily washed into surface water, enters the ground water and aquatic environment in large quantities [1]. On the other hand, the toxicological and epidemiological studies of pesticides in human health concerns are now at forefront due to litany of pesticide residues found in human blood samples, drinking water and foods [2, 3]. Ricceri et al. [4] reported that exposure to CPF at certain gestational or neonatal times of development are associated with neurobehavioral changes. CPF alters synaptic neurotransmission, inhibits neural cell replication, neurite outgrowth, evokes oxidative stress; interfere with signaling cascades and transcriptional events involved in neural cell differentiation [5].

CPF may also regarded as hazardous, since it persists long time in sediments. In India, CPF is classified as an extremely hazardous pesticide [6]; its residue has been found in scented roses and their products [7]. Its maximum concentration has been reported to be 88.6 µg/g in tissues of fishes *Channa striata* and *Catla catla* from Kolleru Lake, India [8] and 198.5 µg/g in sediment, prawn and water samples from prawn ponds near Kolleru Lake wetland [9]. Surprisingly, the soft drinks also contain CPF in a concentration of 4.8 µg/l, which is 47 times higher than permissible limit [10]. Its genotoxicity was reported in *C. punctatus*, mice leukocytes and root meristem cells of *Crepis capillaries* [11, 12, 13, 14].

The measurement of the AChE activities in fish has been suggested as a diagnostic biomarker, with decreased activities indicating water contamination by organophosphorus pesticide [15, 16]. Fish blood is being studied increasingly in toxicological research and environmental monitoring as a possible indicator of physiological and pathological changes in fisheries management and disease investigation [17].The hematological abnormalities under toxic stress may also be reflected in other physiological activities like oxygen consumption and metabolism, which result in death [18].

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Oxygen transport in blood depends upon the hemoglobin content of erythrocytes in blood of fishes [19].

Keeping these facts in view, we have envisaged this study for in-vivo toxic effect of CPF on the activity of AChE in different tissues as well as their tissue specific effects in order to establish the target tissue and their relative efficacy and blood parameters of *C. punctatus*. Finally, our findings would be a useful tool for the control of regional reservoirs and their effective management with respect to the input of CPF from agricultural areas.

[II] MATERIALS AND METHODS

2.1. Chemicals

Technical-grade CPF (20% EC) with trade name Tricel (manufactured by Excel crop care Ltd. Mumbai) was purchased from local market. Trichloroacetic acid (TCA), zinc sulfate ($ZnSO_4.7H_2O$), acetylthiocholine iodide and other chemicals were purchased from Merck. Bovine serum albumin (BSA) for protein assay was purchased from Sigma (U.S.A.).

2.2. Experimental animal

Freshwater fish *C. punctatus* (Bloch) were procured from the local outlets. The fish specimens were an average wet weight and length of 30±2.0 g and 14±3.0 cm, respectively. The fishes were given prophylactic treatment by bathing them twice in 0.05% potassium permanganate ($KMnO_4$) solution for two min to avoid any dermal infections. The fishes were then acclimatized for one month under laboratory condition before CPF exposure. The fishes were fed boiled eggs, goat liver and poultry waste material. The faecal matter and other waste materials were siphoned off daily to reduce ammonia content in water. Every effort as suggested by Bennett and Dooley [20] was made to maintain optimal conditions during acclimatization.

2.3. Determination of sub lethal concentrations

The acute toxicity bioassay to determine the LC50-96h value of CPF in fish was conducted in the semi-static system. A facility for oxygenation of the test solution was provided with the help of showers fixed above the test chambers. The acute bioassay procedure was based on standard methods [21].The stock solution of CPF was prepared by dissolving it in acetone.

A set of ten acclimatized fish specimens was randomly exposed to each of the six CPF target concentrations (0.3, 0.6, 0.8, 1.0, 1.2 and 1.5 mg/l) and the experiment was repeated five times to obtain the LC50-96 h value of the test chemical for C. punctauts.

The LC50-96 h value of CPF was determined as 811.98 µgl-1 for C. punctauts following the probit analysis method as described by Finney [22]. Based on the LC50-96 h value, the two sublethal concentrations of CPF viz., sublethal 1 (1/4th of LC50 = ~203.0 µg/l) and sublethal 2 (1/12th of LC50 = ~ 68.0 µg/l) were estimated.

2.4. In vivo exposure experiment

The fish *C. punctauts* were exposed to the two aforementioned test concentrations of CPF in a semi-static system with the change of test water on every 96 h. The exposure was continued up to 21 days and tissue sampling was done at intervals of 1, 3, 7, 14 and 21 days at the rate of five fishes per duration. The fishes maintained in tap water were considered as negative control. The concentration of acetone was 0.1% in all test solutions and solvent control.

On each sampling day, the blood, gills and brain tissues were collected and immediately processed for estimation AChE and hematological parameters. The blood samples were collected from the fish by caudal vein puncture technique using heparinized syringe. The physicochemical properties of test water, namely temperature, pH, conductivity, dissolved oxygen, chloride, total hardness and total alkalinity were analyzed by standard methods [21].

2.5. AChE and blood parameters analysis

Blood was collected from caudal vein by heparinized syringes from fish C. punctauts and then fishes were sacrificed and tissue like brain and gills were quickly excised in ringer solution. The excess blood was washed with 0.15 M KCl (cold) then weighed. These were homogenized (10% w/v) in 0.1M, pH 8 Tris HCl buffer using homogenizer fitted with teflon pestle. The homogenate were centrifuged at 5000 rpm for 10 min.

AChE activity was determined using the colorimetric technique described by Ellman et al. [23]. The reaction performed at 37ºC was initiated by adding small aliquots of varying concentrations of the substrate (acetylthiocholine iodide) to yield a final volume of 3 ml. The absorbance at 412 nm was recorded continuously for 5 min. The corresponding blanks lacking AChE were substrate to yield the enzymatic activity rate. The typical runs for all experiments used were 2.7 ml buffer, 0.1 M phosphate buffer, pH 8, 50 µl (0.16 mM) DTNB, 100µl (1 mg/ ml protein and 100 µl of substrate. The protein concentrations were measured at 595 nm by Lowry et al. [24], using bovine serum albumin as standard.

Calculation

$$V = \triangle A/\min \times \frac{3}{\text{Protein}} \times \frac{1}{14.3} \ \mu M/\min/mg \text{ protein}$$

Δ A/min is change in optical density.
3 is ml of solution in cuvette.
14.3 molar extinction coefficient of DTNB M/min/mg protein

The blood parameters viz. total erythrocyte count (TEC), total leukocyte count (TLC) were calculated by Neubauer Haemocytometr and Hb%, hematocrit (PCV) by Sahli's hemoglobin meter from collected blood. One aliquot of the sample was used for total erythrocyte count, total leucocyte count, hemoglobin and hematocrit calculations. Chemicals used were of the highest analytical grade and measurements were determined in triplicate.

2.6. Determination of total protein

Protein contents in fish-tissue were determined according to the method of Lowry et al. [24] using bovine serum albumin (BSA) as standard.

2.7. Statistical analysis

The one-way analysis of variance (ANOVA) was applied to compare the mean differences in the AChE levels between tissues within concentration, between durations within concentration and tissue. The different blood parameters were compared between durations within concentration and between concentrations within duration using Mann-Whitney test. P values less than 0.01 was considered statistically significant.

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[III] RESULTS

3.1. Physicochemical properties of the test water

The test water temperature varied from 26.7 to 28.4 ^{o}C and the pH ranged from 7.2 to 8.1. The dissolved oxygen concentration was normal, varied from 6.0 to 8.05 mg/l, during experimental period. The conductivity of the water ranged from 248 to 296 µM/cm and the chloride, total hardness and total alkalinity ranged from 45-54 mg/l, 160-180 mg/land 260-290 mg/l, as $CaCO_3$, respectively.

3.2. Acetylcholinesterase activity and hematological parameters

AChE activity did not differ significantly ($p<0.01$) in solvent and solvent-free control fish. Therefore, the data of AChE activity of solvent and solvent-free controls were combined for statistical analysis. The AChE activity was decreased 51.83% in brain, 44.63% in gill and 39.44% in blood plasma at 203.0 µg/l of CPF **[Figure–1A, Table1]**, while at 68.0 µg/l CPF it reduced 34.86% in brain, 28.52% in gill and 21.60% in blood plasma **[Figure–1B, Table 1]**. Brain AChE level was decreased about half in exposed fish than control.

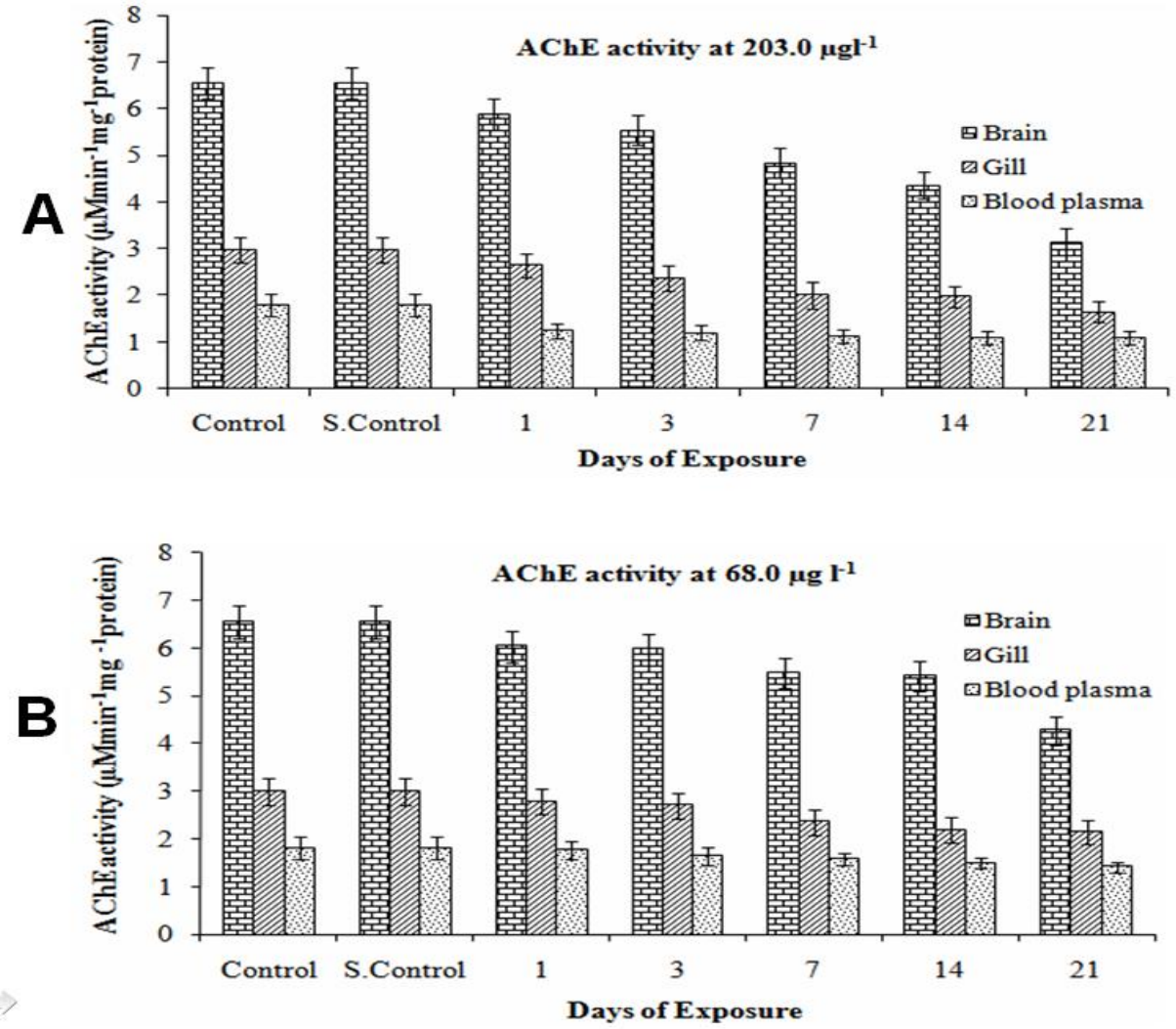

Fig:1. Changes in acetyl cholinesterase activity in brain, gills and blood plasma by **A.** 203.0 µg/l. **B.** 68.0 µg/l of chlorpyrifos.

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Table: 1. AChE activity (µM min/mg/ protein) in different tissues of *C. punctatus* exposed to different concentrations of chorpyrifos at various time intervals

Expo.times	Brain		Gill		Blood plasma	
	203.0 µg/l	68.0 µg/l	203.0 µg/l	68.0 µg/l	203.0 µg/l	68.0 µg/l
Control	6.54±0.34^{a1}	6.54±0.34^{a1}	2.98±0.28^{a1}	2.98±0.28^{a1}	1.80±0.23^{a1}	1.80±0.23^{a1}
S.Control	6.54±0.34^{a1}	6.54±0.34^{a1}	2.98±0.28^{a1}	2.98±0.28^{a1}	1.80±0.23^{a1}	1.80±0.23^{a1}
1 day	5.88±0.33^{a2}	6.03±0.33^{b2}	2.64±0.27^{a1}	2.78±0.28^{a2}	1.24±0.17^{a2}	1.76±0.19^{b1}
3 day	5.53±0.33^{a2}	5.97±0.33^{b2}	2.38±0.26^{a2}	2.70±0.27^{b2}	1.21±0.17^{a2}	1.64±0.18^{b2}
7 day	4.83±0.31^{a3}	5.46±0.32^{b3}	2.01±0.28^{a3}	2.35±0.27^{b3}	1.12±0.14^{a3}	1.57±0.12^{b2}
14 day	4.36±0.32^{a4}	5.40±0.31^{b3}	1.97±0.23^{a3}	2.18±0.27^{b4}	1.10±0.14^{a3}	1.49±0.11^{b3}
21 day	3.15±0.30^{a5}	4.26±0.31^{b4}	1.65±0.23^{a4}	2.13±0.25^{b4}	1.09±0.12^{a3}	1.41±0.11^{b3}

Values with different alphabet superscripts differ significantly (P<0.01) between concentrations within tissue. Values with different numeric superscripts differ significantly (P<0.01) between durations within concentration and tissue.

Blood parameters namely erythrocyte, leukocyte, hemoglobin and hematocrit mean levels decreased significantly (p<0.01) with increase in exposure time and concentration of CPF to the fish **[Figure–2A-D, Table 2]**.

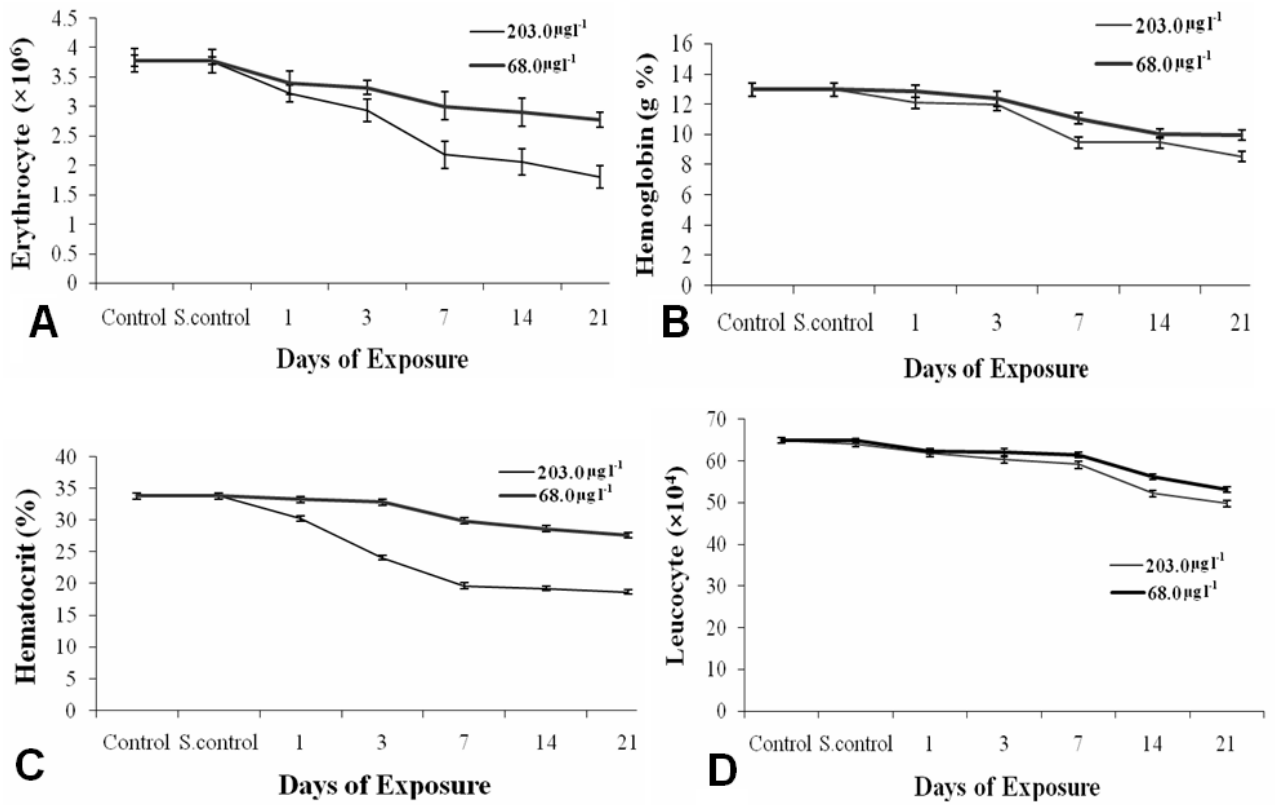

Fig: 2. Changes in blood parameters A. Erythrocyte **B**. Hemoglobin **C**. Hematocrit **D**. Leucocyte after exposure of 203.0 µg/l and 68.0 µg/l of chlorpyrifos for 21days

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Table: 2. Effect of different concentrations of chorpyrifos on hematological parameters of *C. punctatus* at various time intervals.

Expo. times	TEC (×10^5)		TLC (×10^4)		Hb (gm %)		Hematocrit (%)	
	203.0 µg/l	68.0 µg/l	203.0 µg/l	68.0 µg/l	203.0 µg/l	68.0 µg/l	203.0 µg/l	68.0 µg/l
Control	3.78±0.26^{a1}	3.78±0.26^{a1}	64.87±0.63^{a1}	64.87±0.63^{a1}	12.96±0.44^{a1}	12.96±0.44^{a1}	33.80±0.53^{a1}	33.80±0.53^{a1}
S.Control	3.78±0.26^{a1}	3.78±0.26^{a1}	64.82±0.63^{a1}	64.86±0.63^{a1}	12.96±0.44^{a1}	12.96±0.44^{a1}	33.80±0.53^{a1}	33.80±0.53^{a1}
1 day	3.22±0.24^{a1}	3.40±0.25^{b2}	61.87±0.80^{a2}	62.26±0.65^{a2}	12.08±0.39^{a1}	12.86±0.42^{a2}	30.26±0.46^{a2}	33.27±0.51^{b1}
3 day	2.94±0.24^{a2}	3.32±0.24^{b2}	60.20±0.81^{a2}	62.04±0.65^{a2}	11.96±0.38^{a2}	12.34±0.41^{b2}	24.10±0.37^{a3}	32.78±0.49^{b1}
7 day	2.18±0.23^{a3}	3.01±0.24^{b3}	59.10 0.82^{a2}	61.50±0.65^{a2}	9.46±0.36^{a3}	11.04±0.37^{b2}	19.65±0.36^{a4}	29.88±0.49^{b2}
14 day	2.06±0.23^{a3}	2.90±0.24^{b3}	52.23±0.81^{a3}	56.17±0.68^{b3}	9.45±0.36^{a3}	10.06±0.33^{b3}	19.24±0.36^{a4}	28.66±0.48^{b2}
21 day	1.80±0.19^{a4}	2.76±0.22^{b4}	49.76±0.84^{a3}	53.32±0.68^{b4}	8.54±0.34^{a4}	9.90±0.36^{b3}	18.70±0.34^{a4}	27.64±0.41^{b3}

Values with different alphabet superscripts differ significantly (P<0.01) between concentrations within tissue. Values with different numeric superscripts differ significantly (P<0.01) between durations within concentration and tissue.

[IV] DISCUSSION

AChE inhibition was quite high in brain in comparison to other tissues. Natoff [25] reported that increase of acetylcholine at cholinergic synapse resulting from the inhibition of AChE in brain. AChE inhibition process was in two steps due to organophosphate pesticides with the active site of AChE followed by covalent bonding (phosphorylation) of phosphorous of organophosphate pesticide to the oxygen of hydroxyl group of serine [26].

Furthermore, these tissues showed variations in the degree of AChE inhibition for separate treatment and exposure period of CPF. This may be due to differences in the type of interaction between CPF and its metabolites with AChE in various organs, as well as the relative coherence between AChE inhibition and the degree of nerve innervations in these organs. In Tilapia, the highest level of AChE inhibition was noticed in brain followed by kidney, gill [27]. The behavioral changes have been noticed in the form of loss of equilibrium, rapid rate of swimming, convulsions, in the present study, which is in accordance with the findings of Kumar et al. [28] due to neurotoxicity caused by cypermethrin and k-cyhalothrin in *C. punctatus*.

The changes in blood parameters were observed due to long term exposure to CPF in fish. The reduction of erythrocyte count and hemoglobin content in Cyprinus carpio after acute exposure to diazinon were also reported by Svoboda et al. [29].Organophosphate pesticides induce changes in blood parameters, which give evidence for decreased hemotopoiesis followed by anemia induction in fish. Changes in erythrocyte profile induced by acute effect of dichlorvos in Clarias batrachus [30], formothion in *Heteropneustes fossilis* [31], malathion in *Cyprinion watsoni* [32] and trichlorphon in *Piaractus mesopotamicus* [33]. The decrease in erythrocyte number and hemoglobin content observed in this study may be due to the disruptive action of the pesticides on the erythropoietic tissue as a result of which the viability of the red blood cells might be affected. Morgan et al. [34] reported that changes in the hematological parameters were brought about by diazinon as an anemic condition due to decreased synthesis of red blood cells and erythrocyte in bone marrow. Monocrotophos reduced the ventilatory movements and decreased the oxygen intake by impairing neuromuscular transmission through AChE inhibition [35].

The total leukocyte counts (TLC) profile in fish due to CPF exposure showed significant variations. CPF exposure resulted in a significant decrease in the total leukocyte counts that may be due to increased neutrophil leucocytosis. Jain [36] reported that neutrophils are the first line of defense against infections, tissue injury, and parasite attack and in inflammatory response against foreign materials. Svoboda et al. [29] reported a decrease of non-specific immunity in *Cyprinus carpio* after acute exposure to organophosphate pesticides due to decreased leukocyte count. These changes in differential leukocyte count also give evidence for decreased level of non-specific immunity in fish after acute exposure to toxic substances. The adaptation to long term toxicity in relation to lymphocytes could be because lymphocytes are known to play significant role in antigen antibody reaction.

Thus the present study suggest that CPF contaminated water has the potential adverse effect in aquatic organism like fish and further demonstrate the usefulness of the biomarker AChE and blood parameters in an exposure scenario, where it is difficult to get a representative picture of contamination with conventional chemical analysis.

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[IV] CONCLUSION

The technical-grade CPF was found to be neurotoxic to fishes even at lower concentration (i.e.1/12th of LC50 = ~ 68.0 µg/l), which indicates apprehension about the potential hazards of CPF to aquatic organisms. The present investigations indicated that the AChE and blood parameters are sensitive tools for demonstration of toxic effects of CPF in different fish tissues. The results of this investigation may help in guarding against the toxic hazard to human population and the environment through judicious and careful use of this pesticide in agricultural and non-agricultural areas.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

FINANCIAL DISCLOSURE

The work was carried out without any financial support

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CASE REPORT OPEN ACCESS

SUBCUTANEOUS REACTIONS TO IMPLANTATION OF TUBES FILLED WITH AH PLUS AND NEW SEALER

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ABSTRACT

Introduction: *The purpose of this study was to evaluate and compare the subcutaneous reactions of New sealer with AH Plus by subcutaneous implantation in rats as a part of assessment of its biocompatibility.* ***Methods:*** *Twenty seven Wistar rats were divided into three groups of 9 each for observation after completion of 14, 30 and 90 days following implantation respectively. Polyethylene tubes filled with New sealer, AH Plus and tube without sealer (control) were implanted subcutaneously. The sample tissues from sacrificed rats were analyzed histologically.* ***Results:*** *Inflammatory response was graded with FDI criteria as minimal, moderate and severe. Results scrutinized with Student's 't' and ANOVA statistical tests. Inflammatory reaction to AH Plus was moderate at 14 days and minimal at 30 and 90 days, on the contrary, to New sealer it was severe at 14 days and moderate at 30 and 90 days.* ***Conclusions:*** *Inflammatory reaction to AH Plus, in the present study, was moderate at 14 days and minimal at 30 and 90 days. On the contrary, inflammatory reaction to New sealer was severe at 14 days and moderate at 30 and 90 days. The above observations suggests that AH Plus had better biocompatibility at 90 days observation period than New sealer.*

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KEY WORDS

Biocompatibility; AH plus; New sealer

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[I] INTRODUCTION

Several studies have been conducted to assess the biocompatibility of sealers [1-4] essential for ensuring their good performance and success of endodontic treatment. To evaluate the biological response of new endodontic material introduced in to the market, preliminary studies with in vivo experimental material such as implanting these materials in the connective tissue of laboratory animals are commonly performed [4].

It is now appreciated that the sealer has a primary role in sealing the canal [5, 6]. A number of sealers have been formulated in the last several decades [7]. Amongst the characteristics of the sealers used in obturation portrayed by Grossman [8], the most important is that it should be biocompatible i.e. non-irritating to periapical tissue.

Although endodontic sealers are designed to be used only within the root canal, they are frequently extruded through the apical constriction [9] and often placed in intimate contact with periapical tissues for extended periods of time. Thus, it is generally accepted that the biocompatibility of endodontic sealers is critical to the clinical success of endodontic therapy [10].

The large variation in the toxicological and tissue-irritating properties of the materials [11], seems to be not related with whether the tissue is irritated when it comes in contact with the sealer but rather related with what degree and how long it is irritated and hence, it is necessary to evaluate the biocompatibility of these materials for a stipulated period of time.

The methodology to evaluate the biocompatibility parameters comprises of initial tests, secondary tests and usage studies. Subcutaneous implantation of an endodontic material into the connective tissue of rats has been recommended for evaluation of the biocompatibility and the tissue reaction of the material [12]. Friend and Browne [13] concluded that the use of Teflon or polyethylene tubes filled with freshly mixed materials and implanted subcutaneously has greater resemblance to the clinical situation than any other methods.

Resin based sealers have steadily gained popularity e.g. AH Plus is a well established resin sealer. The search for a biocompatible root canal sealer is constant. We have taken new resin based sealer which has been manufactured by Prime Dental Company, India and has not yet been marketed. This

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sealer has not undergone any type of biocompatibility test, which is necessary before its clinical use.

The purpose of this study, hence, is to evaluate the biocompatibility of the New sealer and compare the biological tissue response of the newly developed resin sealer with well established resin sealer AH Plus and to gauge the efficacy and utility of the New sealer in the future.

[II] MATERIALS AND METHODS

Twenty Seven Wistar rats weighing 150-200gm were divided into three groups of 9 each.

Group I – 14 days observation period
Group II - 30 days observation period
Group III – 90 days observation period

In each animal two different materials were implanted at both sides.

Sterilized polyethylene tubes, 10mm in length with1.4mm inner and 1.6mm outer diameters, heat sealed at one end and the opposite end kept open so as to simulate the root canal were used. The New sealer and AH Plus, were mixed according to the manufacturer's instructions and filled in the tubes **[Table–1]**. Material smeared outside the tube was wiped off with the sterile gauze. Empty polyethylene tubes (EPT) were used as control.
The rats were anaesthetized by intra-peritoneal injection of Pento-barbitone sodium (30mg per kg of body weight). With aseptic precautions two pre-prepared polyethylene tubes with different sealers or control tubes were implanted in 15 mm long subcutaneous pockets prepared at two different sites at the inter-scapular area. The two sites of implantation were separated from each other by 20mm to prevent the interference of one sealer from the other [13].

The animals were sacrificed on termination of the experimental periods viz. 14, 30 and 90 days. The skin overlying the implant area was shaved and then the skin including subcutaneous tissue containing the implant was removed along with the surrounding tissue.

The specimen was fixed with 10% formalin and was processed for paraffin embedding. A paraffin block was oriented in such a way that it was parallel to the long axis of the tube and serial sections of 5 - 6 µm were obtained. These were then stained with haematoxylen and eosin.

The slides prepared were thoroughly examined by the two senior pathologists under a light microscope, (Nikon; 40 X), to check the inflammatory reaction. This was a blind assessment without the observer knowing either the length of the observation period or the material tested. The inflammatory response was graded by observing necrosis, inflammatory cell response, vascularity, fibroblastic proliferation and epithelial proliferation (Based on F.D.I. Criteria **Table-2**) [14].

Under 40X microscopic field, cell count was carried out on each section in ten grid fields by using an occulometer grid and results were expressed as average number of cells per grid field.

Tissue response scores were subjected to statistical analysis. To verify its significance Student's't' test and ANOVA test were applied.

Table: 1. Composition of sealers

No	Sealer	Composition	Manufacturer
1	AH Plus	Paste A - Epoxy resin, Calcium tungstate, ZrO_2, Aerosil, Iron oxide Paste B - Adamontone amine, N.N – dibenzyl, 5 – oxananedi amine, 1, 9 – TCD diamine, Calcium tungstate, Zirconium oxide, Aerosil , Silicone oil	Dentsply / Mailleffer, Okla., USA
2	New sealer	Paste A - Epoxy resin bisphenol A Paste B - Aminoethyl ethanolamine, Cocamine ethoxylated	Prime Dental, India

[III] RESULTS

At 14 day observation period, at EPT there was an infiltration of neutrophils, lymphocytes, few macrophages. New blood vessels and fibroblastic proliferation was observed which indicates formation of granulation tissue. This few inflammatory cells, presence of new blood vessels and fibroblastic proliferation indicates mild inflammatory reaction. The presence of inflammatory cells i.e. neutrophils, eosinophils, lymphocytes, macrophages and foreign body giant cells were noted with the New sealer and AH Plus. The fibroblastic proliferation was not seen. The foreign body giant cells (F.B.G.) were observed with engulfed material.

In comparison of Control and New sealer group average number of neutrophils, eosinophils, lymphocytes and macrophages differs significantly ($p < 0.001$) were on higher sides in New sealer. F.B.G.Cells present only in New sealer. In comparison of New sealer and AH Plus, AH Plus showed abundant granulation tissue and was not seen with New sealer. Highly significant number of cells with the New sealer as compared to AH Plus. Lymphocytic infiltration was more with the New sealer. Fibrous

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capsule formation was not seen with New sealer. All above showed a statistical significant difference ($p<0.001$) in neutrophils, lymphocytes and foreign body giant cells and were more in New sealer, these findings are suggestive of severe inflammatory reaction with the New sealer, on other hand, AH Plus showed moderate inflammatory response. **[Figure–1A and -B]**.

Table: 2.The criteria for assessment of tissue response or reactions (Federation Dentaire International Subcutaneous Implantation Test -assessment criteria)

	Mild	Moderate	Severe
2 weeks	The tissue is well organized and no more inflammatory reaction where tissue is exposed to the materials at the end of the tube.	Some inflammatory cells at the open end of the tube. The tissue adjacent to the test material has retained its structure but contains leukocytes [not in remarkable accumulation], lymphocytes, plasma cells, macrophages, occasional Foreign Body Giant Cells.	Distinct tissue reaction at the open end of the tube, fibrous un inflamed tissue along its midsection. The tissue at the open ends of the tube has lost its structure and contains an accumulation of neutrophilic leukocytes & lymphocytes
12 weeks	same as above	Some chronic inflammatory cells like lymphocytes, plasma cells, macrophages, occasional F.B.G. cells at the open end of the tube, with fibrous tissue along the mid section of the tube.	Severe tissue reaction at the open end of the tube.The tissue at the ends of the tube may regained some of its structure but contains some accumulation of – lymphocyte, plasma cells, macrophages, occasional foreign body giant cells [Chronic inflammation]

Note-Continued presence of neutrophilic leukocyte indicates continued tissue disintegration caused by the material

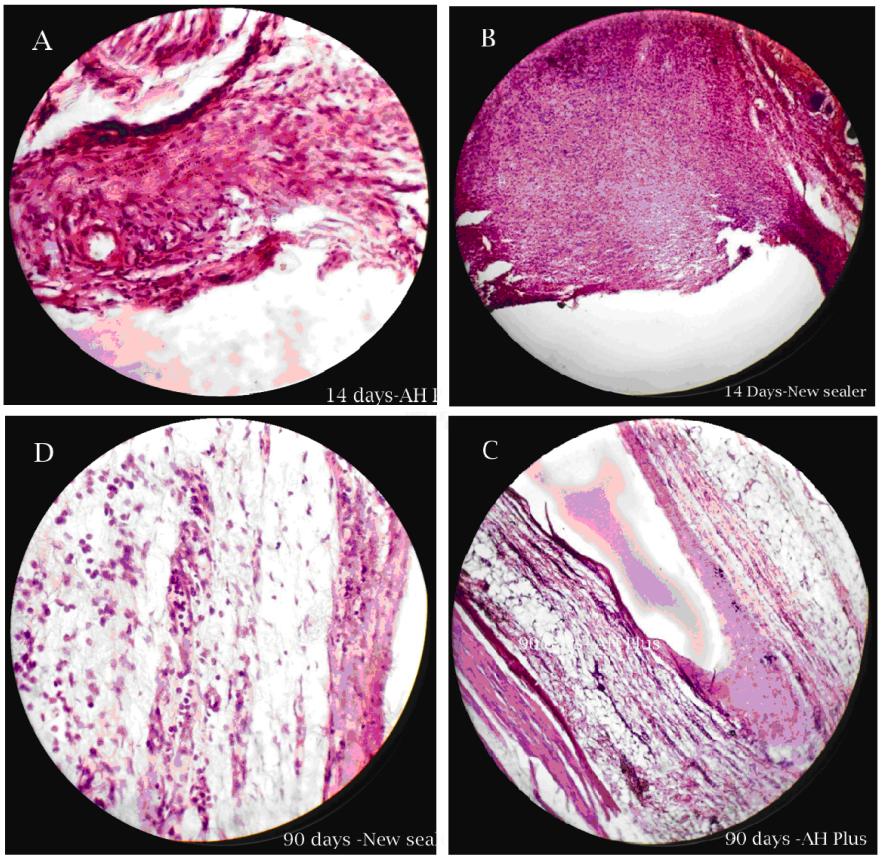

Fig: 1. A) AH Plus, 14 days - Moderate tissue reaction. **B)** New sealer, 14 days - Severe tissue reaction. **C)** AH Plus, 90 days – Mild tissue reaction. **D)** New sealer, 90 Days - Moderate tissue reaction.

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At 30 day observation period the inflammatory reaction was subsided in EPT. Formation of fibrous capsule had started, granulation tissue was becoming avascular. Inflammatory reaction was reduced and neutrophils were absent with both sealers. The AH Plus showed statistically significant difference ($p<0.001$) in cell count as compared to control for macrophages but not ($p>0.001$) for lymphocytes and foreign body giant cells. The New sealer showed a statistically significant difference ($p<0.001$) in cell count for macrophages and lymphocytes but not ($p>0.001$) for foreign body giant cells. The comparison between New and AH Plus sealer showed a statistical significant difference ($p<0.001$) in lymphocytes, macrophages which was more in New sealer.Formation of avascular granulation tissue was more in AH Plus and not seen with New sealer. This shows that inflammatory response was reduced to moderate in New sealer and minimal in AH Plus.

At 90 days observation period, neutrophils were absent in both the sealers. F.B.G.cells were present in New sealer but absent in AH Plus. The comparison between New and AH Plus showed statistical significance ($p<0.001$) for macrophages and lymphocytes which was more in New sealer. AH Plus revealed minimal inflammatory reaction with fibrous tissue formation and F.B.G.Cells were absent. The persistence of chronic inflammatory cell infiltration was noted in New sealer and the formation of avascular granulation tissue and fibrous capsule were not seen. The New sealer had statistically significant response for macrophages, lymphocytes and foreign body giant cells as compared to AH Plus. This indicated persistent irritation of the tissue by the New sealer **[Figure–1C and -D].** Results are summarized in **Table-3 and -4.**

[IV] DISCUSSION

Before introducing a new material in the market, it is fundamental that its properties must be tested. From a biological point of view, its biocompatibility must be evaluated because eventual toxic components present might cause irritation, degeneration, or even necrosis of the tissues adjacent to the material [15, 16]. The biocompatibility of a dental material is an important requirement because the toxic components present in the material could produce irritation or even degradation of surrounding tissues, especially when accidentally extruded into the periradicular tissues [18].

Table: 3. Results at a glance

NO. Observation Period	Control(EPT)	AH Plus	New sealer
1 14 days	Minimal	Moderate	Severe
2 30 days	Minimal	Minimal	Moderate
3 90 days	Complete healing	Minimal	Moderate

Table: 4. Histological tissue response, Differential cell count, 14 days, 30 days and 90 days after implantation

Cells	AH Plus			New sealer			Control		
	Day 14	Day 30	Day 90	Day 14	Day 30	Day 90	Day 14	Day 30	Day 90
Neutrophils	38.33±2.3	0.00±00	0.00±00	48.50±1.3	0.00±0.0	0.00±0.0	16.00±1.4	0.00±0.0	0.00±0.0
Eosinophils	11.67±2.3	0.00±0.0	0.00±0.0	13.00±1.8	6.17±1.3	3.33±0.6	9.33±0.9	0.00±0.0	0.00±0.0
Lymphocytes	20.67±1.7	24.17±3.4	13.33±2.3	29.50±0.9	33.33±1.3	16.83±1.7	13.50±0.9	23.67±1.7	10.33±0.7
Macrophages	6.83±0.6	7.17±0.6	4.33±1.1	8.00±0.8	10.67±1.3	7.50±0.7	4.50±0.5	5.67±0.7	1.83±0.8
F.B.G.Cs.	5.00±0.0	1.00±1.4	0.00±0.0	6.17±0.3	3.67±0.4	3.17±0.8	0.00±0.0	0.00±0.0	0.00±0.0

*The results of the cell counts were expressed as the mean value obtained from the total number of cells which were counted in all specimens of each material (±SD)

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To assess biocompatibility by preliminary 'in vivo' studies, most commonly used is subcutaneous implantation of the material to be studied in small animals [17]. Among these animals, the rats is most frequently used because ,in addition to being an experimental model that satisfactorily represents the body of a mammal, it has adequate dimensions to allow easier and safer management and a more accelerated metabolism when compared to other animal, which allows one to obtain relevant results in a short period of time [27, 17].

Most endodontic sealers are highly toxic when freshly prepared. Their irritating effect increases as material-tissue contact surface area increases [19]. Several studies have evaluated sealer cytotoxicity using in vitro cell culture assays [20, 21], implantation into muscle and peri-radicular response [22]. In vivo tests are based on clinical and histological evaluation of tissue responses. The present study is confined to an 'in vivo' test for evaluating tissue reaction to AH Plus and New sealer by implanting the materials subcutaneously in Wistar rats, the effect of empty polyethylene tube was also studied and compared to the response produced by sealers. The implant test in subcutaneous tissue as recommended by FDI [14] allows the testing of the material as it is utilized in the clinical setup. The implantation of material into subcutaneous connective tissue of rats is considered a suitable secondary test for evaluation of biocompatibility properties of restorative and endodontic materials. This standard practice for biological evaluation of dental materials and their components is recommended before usage test [13, 14]. This method allows for the standardization of the tissue/ material contact area providing the opportunity to compare the biocompatibility of freshly manipulated materials [13].

In the present study, polyethylene tubes were used because of their suitability for maintaining the test materials in contact with the tissue in a controlled manner [23, 24]. Friend and Browne [13] concluded that the use of Teflon or polyethylene tubes filled with freshly mixed materials and implanted subcutaneously has greater resemblance to the clinical situation than any other methods. A small inner diameter of the tube was selected to minimize the flow of material out of the tube and yet allow loading of the sealer. The 10mm of tube length was sufficiently long to have a control surface of side of the tube and the experimental surfaces of the sealer at the open end of tube [13]. The study was done over an observation period of 14, 30 and 90 days. The 14 and 30 day periods were necessary to observe the initial response of the sealers and the 90 day period showed the presence of ongoing inflammation or the resolution of inflammation.

Results were interpreted by preparing histological slides and grading was done, based on F.D.I. Criteria [14], by counting neutrophils, lymphocytes, macrophages, foreign body giant cells and epithelial proliferation, vascularity and collagen fiber deposition.It demonstrated quick healing around the implanted polyethylene tubes by thin fibrous capsules. The reaction was minimal at 14 days as well as at 30 days and showed complete healing at 90 days. Absence of any inflammatory reaction at 90 days confirms the findings of many previous studies that polyethylene tubes can be considered as a good model for animal studies. Torneck [25] has shown similar fibrous tissue repair with no lasting inflammation surrounding the polyethylene tubes.

Microscopically, the inflammatory reaction was observed in AH plus and New sealer. These two sealers were aggressive on the subcutaneous tissue in the beginning. However, though the difference in inflammatory reaction between both the sealers is significant, inflammatory reaction was reduced by 30 and 90 days. Similar responses have been reported in previous studies [26, 13, 27, 22, 28].

The statistical analysis showed the comparison between control and New sealer at 14 days which appeared to be statistically significant for neutrophillic, eosinophilic, lymphocytic and macrophagic response. The New sealer continued to irritate the tissue and hence formation of avascular granulation tissue or fibroblastic collagen synthesis resulting into fibrous capsule formation was not seen, which was observed in the control. This response was less with AH Plus. Comparing control with New material at 30 days, showed statistically significant difference in lymphocyte, macrophage response. The New sealer also showed statistically significant difference, with the presence of foreign body giant cells, whereas this response was not significant with AH Plus as compared to control. At 90 days, only the New sealer had statistically significant response for lymphocytic infiltration and presence of macrophages and foreign body giant cells. The New sealer containing amino ethyl ethanolamine showed lymphocytic infiltration at open end as well as sides of the tube, this response when statistically compared was more in New sealer as compared to AH Plus. The macrophagic response was marked with New sealer as compared with AH Plus.

The foreign body giant cells were observed with the engulfed sealer inside the cells in 30 days and 90 days samples of the New sealer. This indicates persistent irritation of the tissues by the sealer. But on the other hand these cells were seen only in 14 days sample of AH Plus suggestive of gradual decrease in inflammatory reaction. The initial inflammatory reaction may be due to epoxy resin content of the New sealer and AH Plus as well, since, many studies found that several composite resins liberate formaldehyde in amounts sufficient to cause local allergic reaction [17]. This foreign body response was maintained throughout the study period for New sealer unlike AH Plus, suggestive of irritating components in the New sealer, and AH Plus may release formaldehyde from its components in decreasing amounts in aged specimens [29, 30] therefore reducing the inflammatory reaction in later period. The New sealer contains cocamine which is a derivative of coca shrub (Eruthroxylon coca), it has got a local tissue reaction in the form

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of vasoconstriction. This vasoconstriction may be the cause of tissue necrosis in an inflamed tissue, resulting in the exacerbation of inflammation due to New sealer [31]. It also has been demonstrated in one study that water diffusion leads to erosion of composite resin material causing release of unreacted monomers [32]. Hence, the exact cause of persistence of inflammatory reaction due to the New sealer should be investigated, analyzed by further studies to know the exact chemical reaction in the tissues.

In brief, inflammatory reaction to AH Plus, in the present study, was moderate at 14 days and minimal at 30 and 90 days **[Table–3]**. The above observations suggests that AH Plus had better biocompatibility at 90 days observation period than New sealer.

[V] CONCLUSIONS

1) Poor biocompatibility of New sealer was established
2) Severe irritation at 14 days and moderate at 30 and 90 days by New sealer as compared to AH Plus sealer.
3) Cytotoxicity of the individual ingredient of the New sealer should be investigated to find out its chemical reaction occurring at tissue interface resulting in persistence of inflammation.

CONFLICT OF INTERESTS

Authors declare no conflict of interests

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3) The histological analysis was accomplished in the Department of oral pathology and Microbiology, Bharati Vidyapeeth University's Dental College and Hospital, Pune.
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CASE REPORT | OPEN ACCESS

HEREDITARY ECTODERMAL DYSPLASIA- WITH AN UNUSUAL AND USUAL PRESENTATION

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ABSTRACT

Hereditary ectodermal dysplasia is an X linked recessive disorder characterized by defects in the ectoderm. It is characterized by the triad of signs comprising sparse hair, abnormal or missing teeth and inability to sweat due to lack of sweat glands. But it can portray by deformity of at least two or more of the ectodermal structures, hair, teeth, nails and sweat glands. Here we present two case reports of ectodermal dysplasia having hypodontia and hypohydrosis and other classic features of this condition. Radiological manifestations of this condition is less reported which was seen in one of the case report presented as wormian bones. Case reports and review of literature are discussed.

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KEY WORDS

Ectodermal dysplasia anhydrotic (EDA), partial anodontia, sutural bones

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[I] INTRODUCTION

Hereditary ectodermal dysplasia is characterized by defective formation of one or more structures derived from ectoderm. It was first described by Thurnam in 1848 and was coined by Weech in 1929. It is remarkable that no instance has occurred of a daughter being affected [1]. Ectodermal dysplasias (EDs) are a heterogeneous group of disorders characterized by developmental dystrophies of ectodermal structures. The X-linked recessive ED (Christ-Siemens-Touraine syndrome) is the most common disorder (80% of EDs); it affects males and is inherited through female carriers. It is characterized by the triad of signs comprising sparse hair (atrichosis or hypotrichosis), abnormal or missing teeth (anodontia or hypodontia) and inability to sweat due to lack of sweat glands (anhidrosis or hypohidrosis). The lack of teeth and the special appearance were reported to be major concerns. The incidence in male is estimated at 1 in 100,000 births, the carriers-incidence is probably around 17.3 in 100,000 women [2].

A simple attempt made by Nelson included five categories, namely Hypohydrotic (anhydrotic), Hydrotic (Clouston's syndrome), EEC (Ectodactyly ectodermal dysplasia) syndrome, Rapp – Hodgkin syndrome and Robinson's disease [1].

[II] CASE REPORTS

Report-1

This patient was referred to the Department of Oral Medicine and Radiology, College of Dental Sciences, Davanagere, from a private clinic. He presented with partial anodontia. He was of 25 years and lived with this complaint since birth. He gave a history of no eruption of permanent teeth with decreased sweating and increased body temperature. His concern was regarding esthetics and loss of masticatory efficiency. There is no significant family history and medical history. On examination patient had concave facial profile due to depressed malar bone and slightly decreased lower facial height. On intraoral examination there were few retained deciduous teeth resembling canine and molars in the upper and lower arch. Based on these Hereditary Ectodermal Dysplasia was suspected. Verbal consent from the patient was taken for the photographs and radiographs. Intraoral radiographic examination revealed resorbed roots. Extraoral examination revealed sutural bones on the skull radiograph. Sutural bones were seen in the lambdoid

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region. Hand wrist and chest radiograph were normal **[Figure– 1]**.

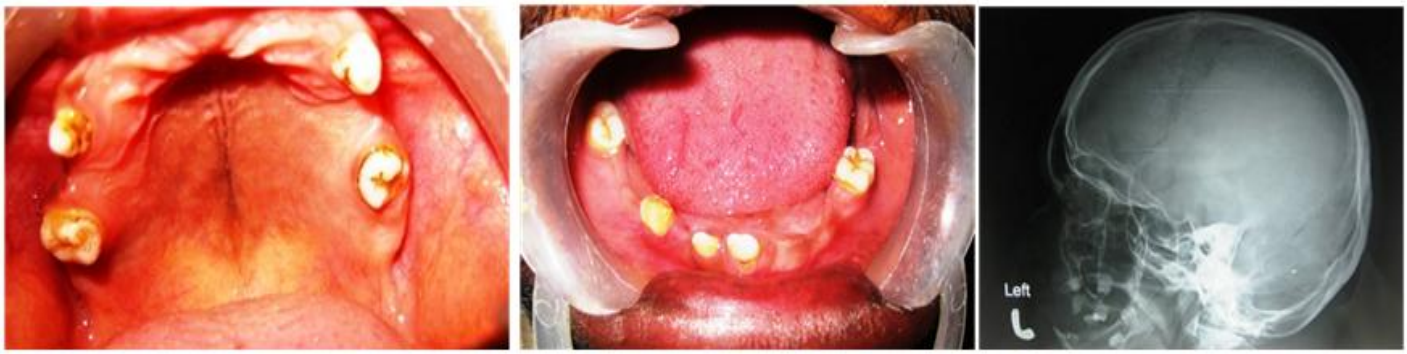

Fig: 1. Clinical photographs revealing hypodontia (several missing teeth) and radiological images depicting presence of wormian bones in the temporal region

Report-2

This patient was referred to the Department of Oral Medicine and Radiology, College of Dental Sciences, Davanagere, from a private clinic. He also presented with partial anodontia. He underwent treatment for his missing teeth but discontinued due to unknown reason and presented to our college for continuation of the treatment. There was no significant medical history. Patient gave history of similar features in the siblings. He gave a history of no eruption of few permanent teeth with decreased sweating and increased body temperature. Verbal consent from the patient was taken for the photographs and radiographs. He also presented with similar features as case 1 with unusual facies and decreased lower facial height. On intraoral examination there were missing lower incisors and upper lateral incisors **[Figure–2].**

Fig: 2. Hypodontia with crown preparation done with upper anterior teeth

[III] DISCUSSION

The prevalence of EDA is unknown; however, the incidence in male is estimated at 1 in 100,000 births although the condition is usually overlooked in infants [2].

Dental anomalies in primary dentition are frequently observed during routine dental examination, leading to orthodontic problems, including spacing or crowding of teeth, loss of arch length, deviation of the midline, increased caries risk, and esthetic problems in preschool children [3].

Hereditary hypohidrotic ectodermal dysplasia is a hereditary disease characterized by deformity of at least two or more of the

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ectodermal structures, hair, teeth, nails and sweat glands. It is typically inherited' as a cross-linked recessive trait so that the frequency and severity of the condition is more pronounced in males than in females [4].

Frontal bossing, usually marked, and depressed nasal bridge give added emphasis to the small size of the face. Due to absence of teeth, and resulting reduced vertical height, the lips are protuberant. The pinnae are often outstanding [5].

A hallmark of this disorder is hypohidrosis. Inability to sweat, because eccrine sweat glands are severely reduced in number, results in intolerance to heat, with severe incapacitation and hyperpyrexia after only mild exertion [5].

The most striking oral abnormality is absence of most deciduous and permanent teeth. Maxillary central incisors and canines usually have a conical crown form and frequently one or more molars may be present these features reported were similar in the case report 1. More rarely, one or both jaws may be edentulous. Female heterozygotes exhibit reduction in numbers of teeth and smaller crown size than hemizygous males. Taurodontism is frequent which were absent in our cases. The alveolar processes do not develop in the absence of teeth [5].

Presence of sutural bones in the skull radiograph is an unusual finding in ectodermal dysplasia. Sutural bones are the intrasutural ossicles commonly seen in lambdoid, posterior sagittal, and squamosal sutures.

Usually represent normal variant, but if extensive then differential includes: PORKCHOPS6

P - Pyknodysostosis
O - Osteogenesis imperfecta
R - Rickets in healing phase
K - Kinky hair syndrome
C - Cleidocranial dysostosis
H - Hypothyroidism / Hypophosphatasia
O - Otopalatodigital syndrome
P - Primary acro-osteolysis (Hajdu-Cheney) / Pachydermoperiostosis / Progeria
S - Syndrome of Downs

The course of the treatment is to restore the function and the aesthetics of the teeth, normalize the vertical dimension and support the facial soft tissues. As long as there are no physical, psychological or social burdens, no treatment is necessary. Early placement of partial or full dentures is commonly recommended from the age of two or three years onwards. The denture must be periodically modified as alveolar growth; erupting teeth and rotational jaw growth change both the alveolar, occlusal and basal dimensions [2].

[IV] CONCLUSION

Even though there are reports concerning this condition with its classical manifestation, reports of sutural bones are minimal or lacking according to our knowledge and why this sutural bones appeared in this condition what the impact it can produce still is inconclusive. But the patient's complaint was taken into consideration and treatment was done appropriately.

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COMMENTARY | OPEN ACCESS

THE DECADE OF OMICS

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[I] COMMENTARY

Etymologically, the suffix -ome originated from the Sanskrit word om implying fullness and completeness [1]. The word genome was first coined by Professor Hans Winkler by blending two words: gene and ome - the latter from chromosome. With the advent of the 'decade of measurements', omics technologies have generated huge amounts of data in different fields of biology from gene sequencing, protein expression, metabolite signatures in organs and molecular pathways in disease amongst others. Currently, the area is expanding as evidenced by the exploitation of the science well beyond genomics into other omics technologies such as transcriptomics, proteomics, pharmacogenomics, nutrigenomics and metabolomics. Henceforth, there is scope for future omics fields to be developed in the post-genomic era of biology and medicine for example at the level of physiology, cell biology and whole organs [2].

In this context, it is worth mentioning that the human genome sequence, along with implementation of novel high-throughput technologies, has empowered us with the blueprint of human health. The knowledge generated by this understanding makes a sound foundation for personalized medicine. Biomarkers based on omics technologies can be used to develop non-invasive diagnostics to identify people at risk of disease. From these, medicine developed using biomedical genomics needs to become a reality through cutting edge research and capability building which will enhance our knowledge of public health and diseases. The underlying pathophysiology of a disease and a patient's response to drugs can also be investigated. Proteomics has already allowed researchers to report and develop a large number of biomarkers from tissue and body fluid.

Currently, transcriptome-based studies on disease prognosis have been used to develop a gene expression-based breast cancer test, namely BCtect® by DiaGenic ASA, Norway, which detects breast cancer at the zero stage or very early by a simple blood test. Similarly, information obtained from pharmacogenomics and nutrigenomics has played a pivotal role not only in the formulation of a new generation of drugs and natural supplements but also in increasing the efficacy and decreasing the adverse effects of current drugs.

The Human Metabolome Project was initiated in Canada in 2005 to identify and quantify hundreds of novel metabolites present in human tissues and fluids with the eventual aim of covering the whole human metabolome. This information will form the Human Metabolome Database and the Human Metabolome Library [3], which will be publicly available in the near future. By 2006, 2200 metabolites had been identified and stored in −80°C for further investigation.

Tanaka in 2010 has postulated the 'three-generation paradigm' to explain the development of omics-based medicine: 'genomic medicine' is the first generation, followed by the '(post-genomic) omics-based medicine' generation and thirdly the 'omics-based systems medicine' generation [4]. This paradigm for molecular medicine works in the following manner: a. 'genomic medicine' allows us to develop tailor-made medicine designed on inborn genome differences; b. '(post-genomic) omics-based medicine' allows predictive medicine designed on both gene expression profiles and protein mass spectra, which are different for different disease stages, and c. 'systems medicine', holistically reveals a detailed understanding of a disease based on cellular pathway changes caused by the disease.

A synthetic biology approach using omics technology has contributed a lot in the preparation of several drug candidates. One example is the production of artemisinic acid, which is an anti-malarial drug precursor in engineered yeast, Saccharomyces cerevisiae [5]. The process yields high amounts of artemisinic acid through the engineered mevalonate pathway, which then resides on the outer surface of the engineered yeast and can be purified in an inexpensive way.

Likewise, in recent years the combination of systems biology, revealing the altered pathways involved in a particular disease condition, and omics has resulted in the development of many leads and some successful drugs, e.g. targeted anti-leukemic

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therapy with imatinib [6], which is a potent inhibitor of the BCR-Abl and the c-Kit tyrosine kinases [7]. The drug generates marked growth inhibition of CML cells and gastrointestinal stromal cell tumors (GIST). Also, the anticancer drug Herceptin is effective for breast cancer patients who over-express the human epidermal growth factor type 2 (HER2) receptor. It is recommended that the diagnostic test for the HER2 receptor must be performed with IHC and FISH for DNA prior to the therapy [8].

Significant developments have been made in the field of mental health, specifically psychiatric genetics. Thousands of scientists worldwide are actively engaged in finding pharmacogenomic and nutrigenomics based nutraceutical solutions to many psychiatric disorders such as psychosis, major depression, bipolar depression, and addictive behaviors (reward deficiency syndrome) among other impulsive and compulsive behaviors. Genome Wide Association Studies (GWAS) are yielding significant results that will ultimately lead to important chromosomal clusters and rare polymorphisms (e.g. SNPs) associated with the entire array of neuropsychiatric and neurological chronic disorders.

Noteworthy advancements have also been achieved in plant, agricultural, pharmaceutical, and industrial biotechnology based on tailor made omics approaches. Completion of Arabidopsis thaliana genome sequence in 2000 [9] and development of high throughput omics technologies over time have stimulated sequencing of various crop genomes including rice and subsequently utilization of these sequencing data for next-generation agri-biotechnology, utilizing integrative omics approaches and metabolic engineering. Successful examples are production of functional foods such as golden rice [10], molecular farming based production of pharmaceutically active agents such as insulin [11], erythropoietin, growth hormones, interferons, peptide vaccines etc [12-14] and various disease specific nutraceuticals [15]. Bioethanol and biodiesel are successful application of the combination of plant and industrial biotechnology [16, 17]. The success in these areas boosted due to the tremendous development of synthetic biology, systems biology, and downstream processing.

The future prospects of omics technologies are understood by developing countries; hence, apart from ongoing, individual work in different laboratories, some dedicated new institutes and centers are working on various disciplines of genomics and proteomics, like the Translational Health Science Technology Institute (THSTI), Faridabad, India; the Institute of Genomics and Integrative Biology (IGIB), New Delhi, India; the International Society of Nutrigenetics / Nutrigenomics, Italy; and the International Society of Psychiatric Genetics, USA.

The Institute of Integrative Omics and Applied Biotechnology (IIOAB)-India, West Bengal is one such organization that has been working since 2008 to provide a global platform for integrative-omics-based multidisciplinary research and advocacy to combat various global challenges. Although, its a non-Govt. organization and does not get any financial support from any source; due to its unique collaborative strategies and mode of research, involvement of multiple top ranked international research groups from interdisciplinary sciences, focused research areas, and high quality research publications; IIOAB has earned good reputation and is now well known to the global scientific community with in this short span of time. Introduction of its official Journal- the ***IIOAB Journal*** is an attempt towards fulfilling IIOAB's commitment in providing an international platform to the scientific community for sharing high quality innovative research works in any area of science, technology, and medicine.

We are highly delighted to become a part of IIOAB's activities and wish to see its continuous shining growth and contribution to the science and our society. We also look forward to publish your research articles, reviews, commentaries, hypotheses and letters to the Editor in the IIOAB Journal.

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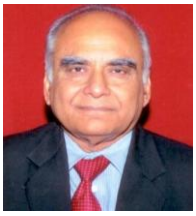

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