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Electrochemical Biosensing of Tea Polyphenols by Modified Enzyme Electrode via Gold Nanoparticles Produced by Green Technology

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Abstract-Metal nanoparticles contribute towards various scientific advancement because of their remarkable physical and chemical properties. Synthesis of nanoparticles via microorganisms could successfully eliminate toxicity, complicate protocol and cost associated with conventional methods. In this paper we had established an eco-friendly, single-step, reagent free extracellular synthesis process using two novel bacteria Pseudomonas alcaligenes RJB-B and Pseudomonas resinovorans RJB-3 isolated from arsenic contaminated soil to produce gold nanoparticles (AuNPs). These nanoparticles exhibited well-defined characteristics and could be used to develop electrochemical sensor for estimation of polyphenols in tea. Bioreduction of chloroauric acid was performed using extracellular media at room temperature to produce 20-50 nm AuNPs. They were morphologically characterized by UV-Visible spectroscopy, transmission electron microscopy, X-ray diffraction, and energy-dispersive X-ray spectroscopy. This method opened an exciting gateway of studying and establishing the role of major biomolecules in extracellular media responsible for bioreduction of salt and the eventual synthesis of nanoparticles. These nanoparticles were stable and could be immobilized easily on glassy carbon electrode along with enzyme tyrosinase under optimum conditions to perform electrochemical tests. Modified electrode gave a rapid and sensitive response for 2.5µM to 110µM catechin equivalent with detection limit of 7.75µM in differential pulse voltammetry (DPV)**.** This work emphasized the importance of biological route for nanoparticles synthesis in a single step with its potential application. The electrochemical sensor developed using the nanoparticles was successfully employed to detect variations in polphenol content of commercial tea samples measured in catechin equivalent and results showed good compatibility with standard method such as HPLC.
Key Words: gold nanoparticles, *Pseudomonas alcaligenes* RJB-B, *Pseudomonas resinovorans* RJB-3, polyphenols, electrochemical sensor, Green Technology

I .INTRODUCTION

In recent times one of the major advancements in nanotechnology and nanoscience studies is the synthesis and application of nanoparticles in biological sciences. Nobel metal (Au,Ag,Pt) nanoparticles synthesis are the most explored area of research due to their wide range of applications starting from daily products (soap, shampoo, detergent, cosmetics etc)[1] to photothermal therapy, water purification and biosensor [2]. Among other metals gold received considerable importance due to its potentially appealing applications spanning a vast vista of fields such as biosensors, biolabeling , catalysis , electronics, optical sensing , drug delivery ,treatment of some kinds of carcinoma and building blocks in nanotechnology[3-8]. There are many established physical and chemical methods for nanoparticles synthesis but they involve certain disadvantages such as elaborate arrangement, instrumentation and use of toxic chemicals. Therefore, development of an eco friendly protocol for nanoparticles synthesis was a big challenge to the researchers throughout the globe considering the environmental issues. Microbes played an important role in green synthesis of nanoparticles. Introduction of environmental pollutants had rendered resistance to various metals and heavy metals in many microbes. As a result tiny microbes became resistant to all kinds of environmental contaminations including metals pollutants. They were also gaining importance because of their use in the area of microbial corrosion, biominerilization, bioremediation and bioleaching. These metal-microbes interaction suggested an alternative method to the scientists for synthesis of nanoparticles in a non-toxic environment [9] Various nanoparticles like silver [10], cadmium sulfide [11], magnetite [12] and gold nanoparticles (AuNPs) had been synthesized using bacteria and fungi.

In this study, we have reported i)a novel single step green synthesis method for production of AuNPs using extracellular extracts of *Pseudomonas alcaligenes* RJB-B and *Pseudomonas resinovorans* RJB-3 ii)a unique biosensor based on modification of glassy carbon working electrode by enzyme tyrosinase and AuNPs produced by the above microbial reduction. This process of reduction of gold salt can be easily carried out at ambient temperature without any addition of costly reagents. *Pseudomonas alcaligenes* RJB-B and *Pseudomonas resinovorans* RJB-3 both are non-pathogenic gram-negative bacteria. These two microbial strains were isolated and reported as arsenic resistant strains by our earlier work [13]. In our present investigation both the bacteria potentially reduced Au(III) at ambient temperature to highly stable Au(0) by extracellular mechanism. It might be interesting to note that extracellular synthesis had several advantages over intracellular synthesis, as it did not involve downstream processes like application of strong detergents or sonication for cell rupture to get

intracellular nanoparticles and can easily be scaled up for large scale production. The exact mechanism of extracellular synthesis of nanoparticles using microbes is still unknown but it is assumed that they might produce different kinds of reducing enzymes that are responsible for nanoparticles formation. To confirm the shape and crystalline nature of the nanoparticles various characterization studies were performed such as UV-spectroscopy, transmission electron microscopy, FTIR (Fourier transform infrared spectroscopy), X-ray diffraction (XRD), and EDX (Energy dispersive X-ray) analysis.

In recent time, electrochemical sensors using enzymes gained remarkable importance because of their amazing performance with high selectivity, good sensitivity and biocompatibility. Applications of nanoparticles in enzymatic sensor contributed towards preparation of more sensitive electrochemical sensor because of their high surface area, ability to facilitate electron transfer rate due to high conductivity and provide better microenvironment for enzyme loading without disrupting their bioactivities. We combined these two aspects in this work to produce an excellent biosensor for polyphenols. Tyrosinase is a copper containing enzyme which also known as polyphenol oxidase. This enzyme catalyzes the o-hydroxylation of monophenols (monophenolase activity) and causes the oxidation of o-diphenols to o-quinones (diphenolase activity). Tea contains major polyphenols belonging to catechin family which provide various health benefits. Till now many research articles highlighted excellent ability of AuNP in biosensing as it can improve communications between enzyme and electrode to get significantly enhanced response. We had successfully utilized the biologically synthesized AuNP with tyrosinase on glassy carbon working electrode assembled with Ag/AgCl reference electrode and platinum counter electrode.

II RELATED WORK

Nanoparticles preparation specially AuNPs synthesis were thoroughly explored using various methods including chemical, physical, microwave and uv irradiation, laser technique etc but all were quite expensive, laborious and sometimes involve toxic chemicals both for preparation as well as for stabilization. Extensive studies and numerous papers had already proved biological route as a promising methods with wide range of potential applications [15, 16]. Gold is one of the most promising and preferred noble metal in compare to other metal. Synthesis of AuNPs using biological methods is a clean, regent less approach as chemical methods include certain disadvantages like use of costly chemical for synthesis and stabilization which are also toxic in nature. These are the main reasons why we decided to explore gold nanoparticle synthesis using a biological method. An earlier report of Beveridge and Murray [17] on AuNPs synthesis stated that Bacillus subtilis cells when incubated with gold chloride solution formed nanoparticles. Some well-known examples of AuNPs formation utilizing different kinds of microorganisms are *Fusarium oxysporum* [18], *Sargassum wightii* [19], *Lactobacillus sp*. [20], *Pseudomonas aeruginosa* [21], *Rhodopseudomonas capsulate* [22], and *Bacillus thuringiensis* [23]. Some of these approaches included extracellular as well as intracellular synthesis of nanoparticles. We preferred extracellular synthesis over intracellular because of certain advantages: i) no toxic chemical used and hence green route ii) no downstream processing (ii) highly reproducible (iii) easy maintenance of culture (iv) cost effective and (v) highly stable under normal conditions. There are no reports in the literature on synthesis of AuNPs using arsenic resistant novel bacteria *Pseudomonas alcaligenes* RJB-B and *Pseudomonas resinovorans* RJB-3.

Over the past decades, several reports describing working mechanism of tyrosinase had been published. This enzyme has been extensively used in the development of sensors for the phenolic compounds [24]. Several other methods like spectrophotometry, various chomatographic techniques (e.g. HPLC) and electrophoresis were some of the methods generally employed for analysis of phenolic component in various samples [25]. Though some of these methods are quite efficient in analyzing polyphenols, their applications are quite limited due to requirement of expensive chemicals, elaborate sample preparation, long operation time and trained personnel for handling instrument. Electrochemical sensors have established advantages over the conventional methods. Its construction is simple, low cost, highly selective, gives rapid response and requires minimal preparation of sample. Differential pulse voltametry, cyclic voltametry, and amperometry are different electrochemical methods already employed for polyphenols analysis. We preferred to use DPV as this is the most efficient analytical methods for accurate identification as well as quantification of polyphenols in real samples. The proposed work successfully utilized an enzyme electrode modified with AUNPs to develop a cost effective sensor for biotechnology, biomedical and industrial use. This report is the first of its kind where biologically synthesized nanoparticles have been used to develop an electrochemical sensor of polyphenols.

III MATERIALS AND METHODS

A. Chemicals and Reagents

Hydrogen tetrachloroaurate tri hydrate ($HAuCl_4$, $3H_20$) was purchased from Himedia and used as received. Phosphate buffer was prepared with 0.2M Na_2HP0_4 and NaH_2P0_4 obtailed from Sigma (USA). Catechin wasis purchased from Sigma (USA). Microbiological media and ingredients were obtained from Hi media (India). All other ingredients used in this experiment were obtained from Merck, Germany. Ultrapure Millipore water (18.2Ω) was used throughout the

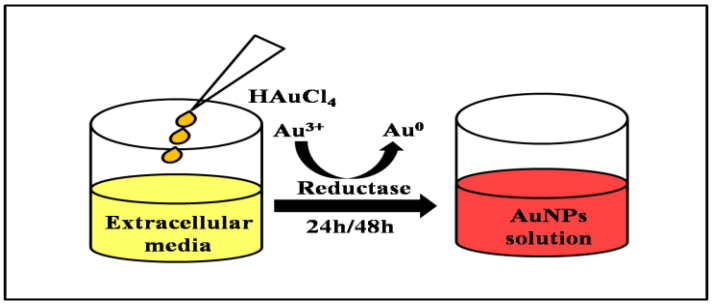
experiments. Tea samples of commercial brands were purchased from local market.

B. Instrumentation

Transmission electron microscopy (TEM) was done using TECNAI G2 30ST, FEI Company (Netherlands) equipped with EDX analyzer (EDX Inc., Mahwah, NJ). XRD measurement was conducted by XPERT-PRO diffractometer system. To understand bioreduction of gold chloride in extracellular solution and formation of AuNPs, UV-visible spectroscopy has been performed by Cecil Aquarius double beam spectrophotometer (model no CE 7200) with milliQ water as reference. HPLC analysis for tea polyphenols was carried out by a Varian LC system (USA) equipped with a ProStar 230 solvent delivery module, and a ProStar 330 PDA detector. The separation of phenolic compounds was performed on an OmniSpher C18 column (25 cm x 4.6 mm, Varian, USA) equipped with ChromSep guard-column (1 cm x 3 mm, Varian, USA). Electrochemical measurements i.e. DPV was carried out with Autolab PGSTAT-30 using GPES (general purpose electrochemical system) software. Three electrode systems were assembled using glassy carbon as working electrode, Ag/AgCl as reference electrode and platinum coil as counter electrode in phosphate buffer (pH 7) as a supporting electrolyte.

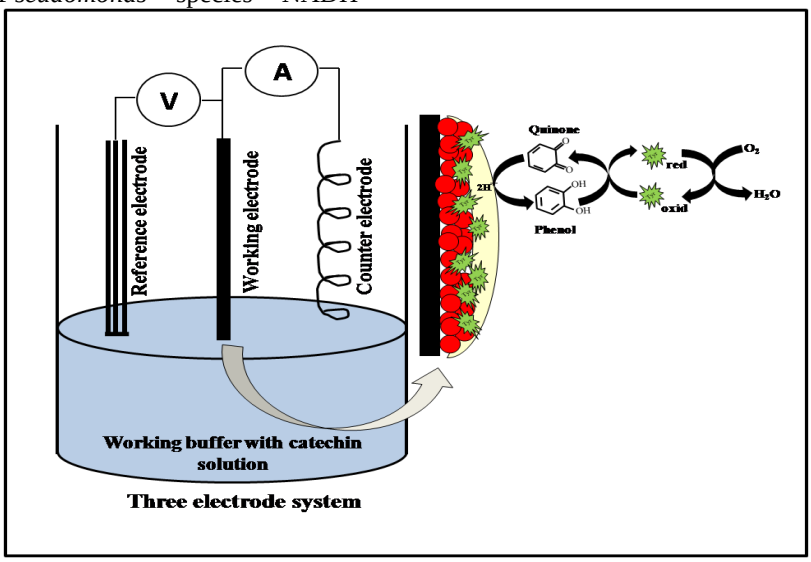
C. Methodology

Extracellular synthesis of AuNPs indicated presence of reductase enzyme in the cell free extract which was released by two bacteria in the media and these enzymes were mainly responsible for reductions of Au^{3+} ions to Au^{0}. Among many reductase enzymes in *Pseudomonas* species NADH dependent reductase enzyme is the most common enzyme in cell free extract which was already reported for reduction and formation of AuNPs from gold salt (Scheme 1). The reduction takes place due to electron shuttle between NADH to NAD^{+} and Au^{3+} to Au^{0} where NADH dependent reductase works as an electron carrier. The nanoparticles produced by the proposed synthesis process were highly stabilized by the proteins in the media and no additional stabilizing agent was needed.



Scheme1. Schematic presentation of possible mechanism behind formation of AuNPs

We used these nanoparticles to develop enzyme based electrochemical sensor for polyphenols. The principle of redox reaction is given in Scheme 2. The polyphenols are oxidized to corresponding quinone on AuNPs modified glassy carbon electrode. The depletion of oxygen level involved consumption of electrons during this reaction and as a result an electrochemical signal was generated and this was directly proportional to the polyphenol concentration in the solution. AuNPs helped in electron transfer between enzyme and electrode resulting an improved response and hence higher sensitivity.



Scheme 2. Schematic presentation of reaction pathways in electrochemical sensor using three electrode system

D. Preparation of extracellular extracts of bacterial strains

The metal resistant strains *Pseudomonas alcaligenes* RJB-B and *Pseudomonas resinovorans* RJB-3 were isolated from soil sample collected from arsenic contaminated area in Chakdah, North 24 Parganas, West Bengal, India (23°39' N, 88°35' E). Both the bacterial strains were grown in nutrient agar plates (containing 0.5% peptone, 0.5% NaCl, 0.2% yeast extract, 0.2% beef extract and 1.5% agar; pH 7), incubated at 37 °C for 24 hours. The details of the isolation, identification and culture maintenance were described in our earlier work [13]. Accession numbers FJ866633 and FJ866631 were assigned for the strains RJB-B and RJB-3 respectively by NCBI Genbank on submission of 16s rRNA gene sequencing generated by automated DNA sequencer (MEGA BACE 1000, GE Healthcare, USA). Both the bacterial strains were grown in 100ml nutrient broth and incubated at 37ºC overnight in a rotary shaker (150 rpm). Cell free extracts were then collected by centrifugation (3000 rpm for 10min) after overnight incubation.

E. *Extracellular biosynthesis of gold nanoparticles*

Cell free extract from both RJB-B and RJB-3 were added separately with chloroauric acid ($HAuCl_4$) to make the final concentration of 1mM of gold. A control system included chloroauric acid of same strength in 10 ml nutrient broth media without cell free extract. Both the control and test media were allowed to incubate at 37ºC in a rotary shaker (200 rpm speed). After 24 hours of incubation, cell free media containing biosynthesized AuNPs were obtained and the colour of the reaction media changed from pale yellow to wine red whereas colour of the control media remained unchanged**.** Immediately, the AuNPs were collected by ultra centrifugation, washed thrice with MilliQ water to remove extracellular media. The nanoparticles thus obtained were stored as a stock solution in doubly distilled water at 4ºC in dark before further use. Various characterization studies were conducted on these biologically synthesized AuNPs.

F. Characterization of gold nanoparticles

To understand bioreduction of gold chloride in extracellular solution, UV-visible spectroscopy was performed from 400 to 900nm with milliQ water as reference. Morphology of AuNPs such as shape and size of the particles were observed with (TEM) on a carbon coated copper grid at a voltage of 120kV. XRD measurements are done at 40 kV to investigate lattice structure of nanoparticles.

G. Enzyme electrode preparation

A three-electrode system was used for development of tea polyphenol sensor. This three-electrode system was composed of glassy carbon as working electrode (WE), Ag/AgCl as reference electrode (RE) and platinum coil as counter electrode (CE) in phosphate buffer pH 7 as a supporting electrolyte. Bare glassy carbon electrode was washed with micron aluminum oxide powder before enzyme immobilization. It was then sonicated with ethanol/water to get rid of residues of alumina. After cleaning the surface of WE, 5 µl of AuNPs solution was deposited on the surface of the glassy carbon electrode and left in air to dry. Then 10 µl of pure tyrosinase solution from stock (500U/100 µl) was dispensed. Lastly, 10µl of 20% gelatin and 5µl of 12.5% glutaraldehyde were deposited to immobilize the enzyme and AuNPs. After drying, immobilized enzyme electrode was washed with phosphate buffer pH 7 to remove all unbound reagents from the surface and stored in 4ºC before use.

H. Electrochemical measurement

Differential pulse voltametric measurements were carried out using AUTOLAB PGSTAT-30 with GPES software. To monitor enzymatic reaction scanning was carried out between -0.05V to 1.0V with scan rate of 30mV s^{-1} by differential pulse voltammetry. This scan rate 30mV s^{-1} was the optimized value since best response was obtained at this scan rate under optimal experimental conditions e.g. temperature, pH, experiential time. To construct a calibration curve, we used catechin as standard polyphenol and experiments were conducted by registering the DPV response for various concentrations of catechin in 6ml of working buffer (phosphate buffer pH 7). Each experiment was performed three times to avoid experimental errors. All electroananlytical measurements were performed at room temperature.

I. Preparation of tea samples and electrochemical measurement

After constructing the calibration curve of standard catechin, the amount of polyphenols present in tea samples was also measured and expressed as mg catechin/g of tea leaves. For this purpose, all the tea samples were bought from local market (1 CTC grade, 1 oolong tea, 1 black tea and 2 green teas). 10mg of each sample was weighed and brewed in 20ml of hot water for 15 minutes. The tea infusion was filtered out and cooled to room temperature before measurement electrochemical biosensor.

J. HPLC measurements

The total polyphenol content of the tea samples were determined by comparing with peak areas obtained for the samples vis-à-vis peaks given by standard solutions of a number of polyphenols in the catechin family such as gallic acid, epigallocatechin, (+)-catechin-, caffeic acid, (-)-epicatechin, catechin gallate. The individual peak area given

by HPLC chromatograms at 280 nm corresponding to the individual members of catechin family was calculated and summed up to get total polyphenol.

[Table 1]

IV. RESULTS AND DISCUSSION

The cell free extracellular nutrient broth media when mixed with an aqueous $HAuCl_4$ solution slowly turned wine red from pale yellow (Fig. 1). The red colour was due to excitation of surface plasmon vibrations in the AuNPs. On the other hand, the control media remained pale yellow in colour.

Fig. 1

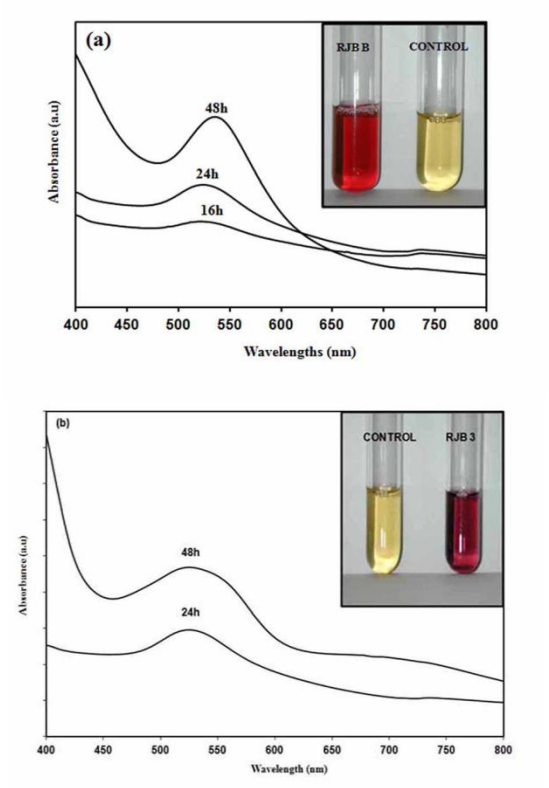


Fig. 1(a)
Fig. 1(b)

Fig. 1 UV-visible spectra of gold nanoparticles from cell free extract as a function of time
(a) Spectrum obtained from *Pseudomonas alcaligenes* RJB-B Inset shows test tube containing HAuCl4 without any extracellular media marked as control and with extracellular media marked as RJB B
(b) Spectrum obtained from of Pseudomonas resinovorans RJB-3. Inset shows test tubes containing HAuCl4 without any extracellular media marked as control and with extracellular media marked as RJB 3

Reduction of aqueous solution of $HAuCl_4$ by extra cellular medium of bacteria was observed with time by UV-visible spectrophotometry, the most common and primary approach in characterization on any nanoparticles. The UV-visible spectra showed a sharp absorbance spectrum centered around 540nm which steadily increased with time. This indicted that biologically synthesized particles uniform in size and there were not much aggregations. There was no visible peak in the control solution as expected. Interestingly, the gold particles thus produced were found to be stable for more than 2 months with very little aggregations in the solution. The reason behind stability of the nanoparticles might be that the proteins present in cell free extract worked as a capping agent for biofabricated AuNP. The spot profile of EDX (Fig. 2) obtained for both the samples showed prominent signals of gold.

Fig. 2

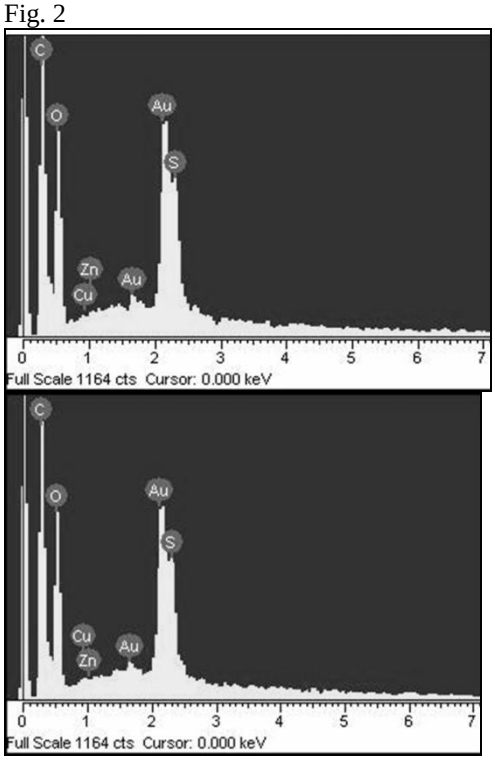


Fig. 2(a)
Fig.2(b)

Fig. 2 EDX spectrum of gold nanoparticles synthesized by (a) *Pseudomonas alcaligenes* RJB-B (b) *Pseudomonas resinovorans* RJB-3

Shape and size distribution of the particles were determined by TEM study (Fig. 3) which revealed that most of the nanoparticles were well separated, uniform and spherical in nature with an average size of 20-40nm.

Fig.3

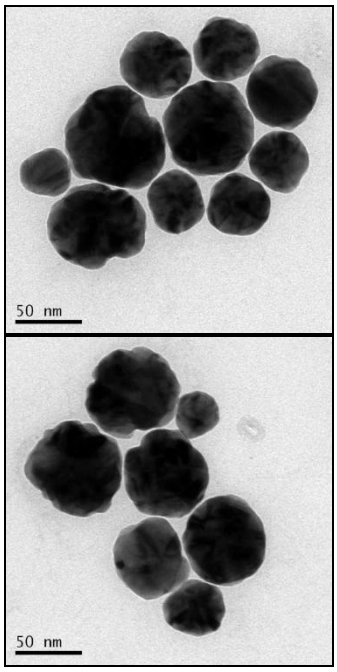


Fig. 3(a)
Fig. 3(b)

Fig. 3 TEM micrograph of gold nanoparticles obtained by bio-reduction of HAuCl4 with extracellular media
(a)Micrograph of AuNPs obtained from Pseudomonas alcaligenes RJB-B
(b) Micrograph of AuNPs obtained from Pseudomonas resinovorans RJB-3

Crystalline nature of AuNPs was further confirmed from XRD pattern of dried biofabricated colloidal gold solution (Fig. 4).

Fig. 4

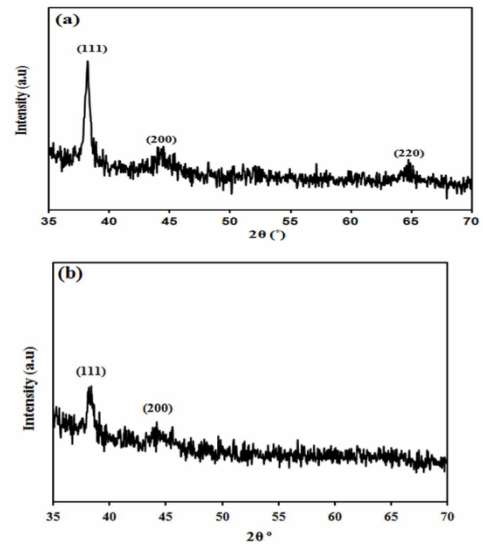


Fig. 4(a)
Fig. 4(b)

Fig.4 XRD spectrum of dried gold nanoparticles synthesized by (a) *Pseudomonas alcaligenes* RJB-B (b) *Pseudomonas resinovorans* RJB-3

Results showed three intense diffraction peaks in case of RJB B (Figure.3a) at $2\theta = 38.19, 44.29$ and 64.71 which could be indexed to (111), (200), (220) on the basis of face centered cubic (fcc) gold structure which was similar to already reported standard gold metal(Au^0). Figure 3b also showed XRD pattern obtained from RJB 3, which indicated two intense peak at $2\theta=38, 44.2$ corresponding to (111), (200) Bragg's reflection based on fcc structure of AuNP. The peak corresponding to (111) plane was more prominent than others, which highlighted the fact that the (111) plane was dominant crystallographic plane. It was also interesting to note that the surface of the AuNP were evidently flat and crystalline in nature. Absence of any other prominent diffraction peaks reduced chances of impurities in the samples.
We used DPV as a quantification method for catechin detection because it is one of the most efficient tools for peak to peak separation of any analytes. Catechin is one of the most important polyphenolic compounds among other 7 major polyphenols in tea. Fig. 5 showed DPV response for different concentrations of catechin in phosphate buffer (pH 7) at 30mV s^{-1}.

Fig. 5

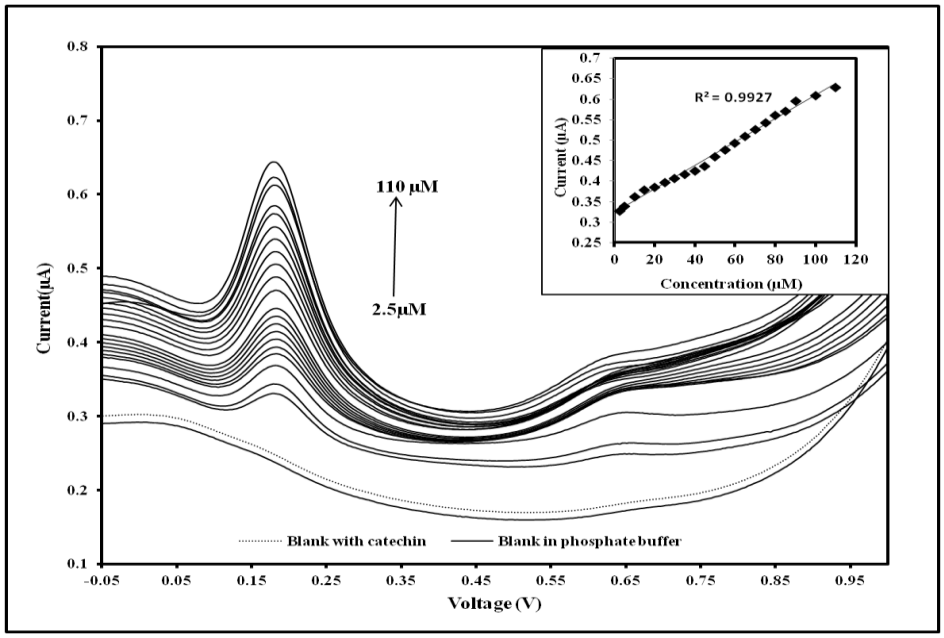


Fig.5 Differential pulse voltammetry graphs of catechin using Tyr-AuNP/GC electrode in the presence of different concentrations of catechin: 2.5µM to 110µM at the scan rate of 30mVs^{-1}. Inset was showing calibration plot of catechin solutions in µM vs. peak height in µA

Results of DPV showed one prominent oxidation peak at 0.16V responsible of enzymatic oxidation to corresponding quinone. Enzyme electrode in buffer or bare electrode with catechin in buffer did not show any prominent peak. Increase in anodic peak current with increased concentration indicated higher rate of enzymatic oxidation. Only a single electrode was used to study influence of different concentrations of catechin from 2.5µM to 110 µM with detection limit of 7.7 µM which was quite low with a signal to noise ratio (S/N=3) and good correlation coefficient 0.9927. We used goldnanoparticles from RJB B for sensor preparation because of it gave uniform round shaped nanoparticles. The AuNPs acted both as a biocompatible environment and an efficient promoter to improve enzymatic kinetics. Electrode was always stored in 4°C when not in use. When stored for 15 days in 4°C, the immobilized electrode showed no visible change in DPV response. Covalent attachment of enzyme tyrosinase on the GC electrode with crosslinker glutaraldehyde and gelatine might be responsible to prevent leaching of enzyme from the working electrode. We analyzed 5 commercially available teas of different categories including green tea, black tea and CTC grade (Fig 6) under optimized conditions.

Fig. 6

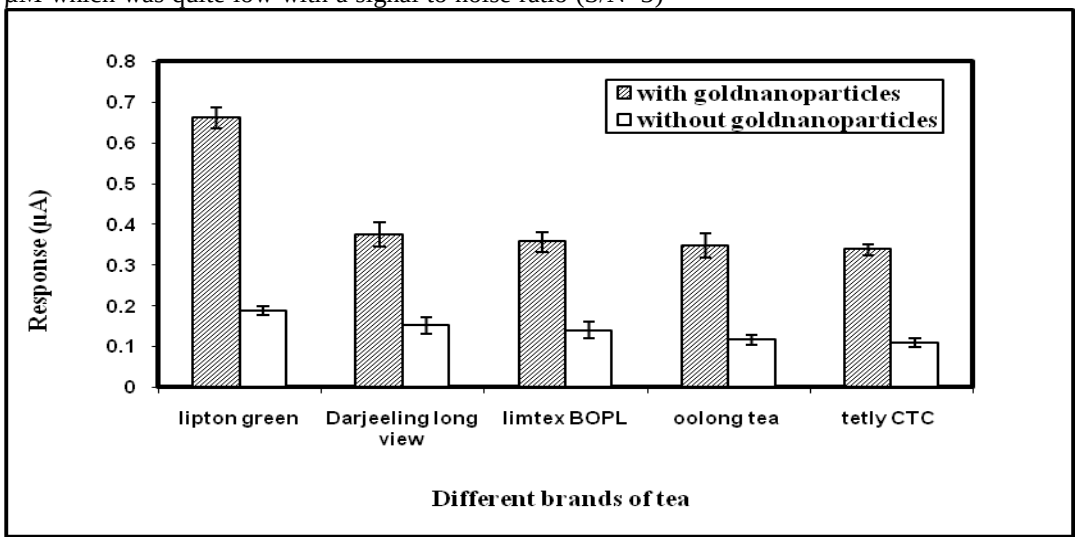


Fig.6 Analysis of five commercially available tea with and without AuNPs modified enzyme electrode under optimized conditions

Comparison of the responses obtained from tea samples showed that the presence of AuNPs increased responses considerably. These results confirmed presence of nanoparticles helped improve electron transfer rate and hence current responses. Polyphenolic content measured by present sensor was also compared with standard HPLC analysis (Table 1). The HPLC values were less than the values obtained by the electrochemical sensor since only catechin group could be analyzed by HPLC whereas the sensor gave response to all the polyphenols present in tea. The results seem to be very satisfactory since a better prediction could be possible by the our developed electrochemical system when compared with very costly (HPLC) but authentic results.

Table1. Comparison between different brands of tea using electrochemical method and standard HPLC method

Samples	Total polyphenols content using electrochemical method in µM (Mean ± SE, n=3)	Total polyphenols content by HPLC method (Mean ± SE, n=3)
Tea		
(i) Lipton green	95.51 ± 0.025	85.2 ±0.003
(ii) Darjeeling longview	18.05 ±0.029	15.1±0.004
(iii) Limptex BOPL	11.53 ±0.023	9.23± 0.008
(iv) Oolong	8.69 ±0.029	7.21± 0.003
(v) Tetly CTC	5.11±0.014	4.5±0.005

Note SE=standard error

Conclusion

An efficient synthesis of AuNPs by successful reduction of gold chloride was carried out by two bacteria *Pseudomonas resinovorans* RJB-3 and *Pseudomonas alcaligenes* RJB-B isolated from arsenic contaminated soil in the laboratory for the first time. The method could be considered as a green technology since no toxic reducing agent was used for reduction and the process was carried out at ambient conditions. Though the exact mechanism behind nanoparticles formation is still not understood well, the reducing enzymes secreted by microbes present in extracellular media could play active role in the reduction process. The synthesized AuNPs were found to be of uniform shape and size and hence could be used for enzyme activity enhancer for biosensing purpose. An electrochemical biosensor was developed and this could give polyphenol content in various tea samples in mg catechin/g tea leaves. The method utilizing AuNPs improved responses of ordinary enzyme sensor. The results when compared with that of HPLC gave excellent match and in fact a better one and thus could safely used for gradation of tea based on the total polyphenol content. The work has two distinct contributions: i) a green efficient route of production of AuNPs ii) construction of polyphenol biosensor for proper gradation of tea.

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