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# Response surface analysis of nano-ureases from *Canavalia ensiformis* and *Cajanus cajan*

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ABSTRACT

Ureases isolated from leguminous sources, *Canavalia ensiformis* and *Cajanus cajan* were immobilized onto gold nanoparticles (nano-ureases). Optimization of the urease immobilization was carried using response surface methodology based on Central Composite Design. Immobilization efficiency of nano-urease from *C. ensiformis* and *C. cajan* were found to be 215.10% and 255.92%, respectively. The methodology adopted has deviation of 2.56% and 3.01% with respect to experimental values in case of *C. ensiformis* and *C. cajan*, respectively. Nano-urease from *C. cajan* has broad physico-chemical parameters with pH optimum from 7.1 to 7.3 and temperature optimum from 50 to 70 °C. Nano-urease from *C. ensiformis* has sharp pH and temperature optima at 7.3 and 70 °C, respectively. Fourier transform infra-red spectroscopy has revealed involvement of groups *viz*. amino, glycosyl moiety, etc. in urease immobilization onto gold nano-particles. Transmission and scanning electron micrographs revealed that arrangement of urease onto gold nano-particles from *C. ensiformis* was uniform while it was localized in case of *C. cajan*. Nano-urease from *C. ensiformis* has higher specificity and catalysis toward urea as compared to nano-urease from *C. cajan*. Nano-ureases from both sources are equally stable for 6 months under dried conditions and can be used for 10 washes.



## 1. Introduction

Urease is known as an enzyme to hydrolyze urea into ammonia and carbon dioxide. The enzyme is used in diagnostics to determine urea present in the blood serum. It has become possible now to decompose urea using artificial kidneys containing immobilized urease (Japanese Patent Publication Nos. 36751/85 and 17467/86). The urease from plants plays a crucial role in their protection against insects by damaging anterior midgut [1]. In food industry, urea has been marked as an undesirable substance, particularly in biologically fermented food products such as sake, beer, wine, soy sauce, etc. Presence of urea in food products gives bitter taste, cause coloring as well as deterioration of the flavor, thus lowers the quality of food. Urease is being used for treatment of waste water plant particularly from agriculture, rich in urea [2]. Urea hydrolysis by urease increases the pH of the environment leading to calcification, which has numerous biotechnological applications. These include preparation of advanced carbonate materials, which are used as sealant for cementing surface cracks in building and historic monuments. The bio-precipitation of $CaCO_3$ is also used as a plugging agent in oil reservoirs by decreasing the permeability of porous areas of the reservoirs, helpful for enhancement of secondary and tertiary oil recovery [3,4]. For these applications, neutral urease (pH optimum at neutral) obtained from seeds of plant of bean species such as *Canavalia ensiformis*, or from micro-organisms such as *Eurobacterium aerofaciens* and *Proteus mirabilis* are practically used, and good results have been obtained thereby. The approach is still going on to explore more and more cheap source of urease, which are readily accessible and with better physico-chemical properties.

The practical utilization of any enzyme is driven by its versatility, regio-, chemo- and enantio-selectivity while operating under mild conditions making any process environmentally compatible. As enzymes are sensitive, unstable and have to be used in water, they are still in their puberty stage to be industrially implemented. Enzyme immobilization is one of the best ways to avoid these complaints and further it is useful for continuous processes. Even processes employing insoluble enzymes suspended in hydrophobic media also requires immobilization to optimize enzyme dispersion to improve accessibility for the substrates, as well as to avoid

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their aggregation [5]. The anchoring of an enzyme onto a solid insoluble support should be straightforward and cost-efficient. The key factor determining the success or failure of immobilization depends on the methodology adopted. Most important factors are the size and shape of the matrix used for enzyme immobilization [6]. One of the most serious problems for the industrial application of the technology of immobilized enzymes is the diffusional resistance to transport of substrates [7]. This limitation arises from steric hindrance of the solid matrix to the free diffusion of substrates and products toward or away from the catalytic site of immobilized enzymes [8]. During recent years, nano-particles have gained much significance in variety of fields, *viz*. agriculture, medicine, veterinary, etc. It is one of the most desirable matrices for enzyme immobilization due to its very small size, it minimizes diffusional constrains and provides large surface area for enzyme attachment. Urease immobilization is one of the most-effective ways for its various industrial applications which imparts stability and improvement in physico-chemical parameters [9–12]. Urease immobilization onto various nano-particles like titanium oxide, iron oxide, glycidyl methacrylate grafted sodium alginate, etc. have tremendously increased the stability of enzyme but there is negligible increase in the catalytic efficiency of enzyme [13–15]. The present work is based on urease (from *Canavalia ensiformis* and *Cajanus cajan*) immobilization onto gold nanoparticles (AuNps) using covalent linkage, *via* cysteamine-glutaraldehyde tail attached to them. Further, we have studied various physico-chemical changes brought up by immobilization.

Experimental design is regarded as the most favorable technique, which covers a large area of practical statistics and obtains definitive results with the least expense [16]. Response surface method (RSM) is one of the popular experimental designs help to quantify the relationships between one or more measured responses and the vital input factors. The present work is based on optimization of urease immobilization onto AuNps using central composite design (CCD). CCD involves full factorial search by examining simultaneous, systematic and efficient variation of important components, identifying possible interactions, higher order effects and determining the optimum operational conditions. However, RSM using CCD is useful for a small number of variables (up to five) but it is impractical for a large number of variables due to the high number of experimental runs required. CCD used here is based on two variables at three levels using percent immobilization efficiency as the response.

## 2. Experimental

### 2.1. Chemicals and enzyme

All the chemicals for buffers and other reagents were of analytical grade or electrophoresis grade. Unless stated all the chemicals were purchased from Sigma (St. Louis, MO, USA). Milli Q water with resistance of greater than 18 MΩ/cm (at 25 °C) was used throughout the experiments. Urease from *C. ensiformis* was bought from Sigma (St. Louis, MO, USA) while urease from *C. cajan* was extracted and purified according laboratory protocol by Das et al. [17].

### 2.2. Synthesis of AuNps

The AuNps were prepared by reducing the solution of $HAuCl_4$ with lemon extract. The lemon extract was prepared from the juice extracted from peeled off lemons followed by centrifugation ($1000 \times g$) and dilution with Milli Q water in the ratio, 1:1. The $HAuCl_4$ salt was dissolved in 50 mL of lemon extract (4 mM), which was added to $HAuCl_4$ solution drop by drop and stirred on a magnetic stirrer for 3 h. The solution was ultra-sonicated at high frequency of 10 kHz for 1 h and then kept at stationary stand-still position for 2 h at room temperature. The solution turned red, indicating the precipitation of AuNps. The precipitate was filtered out, and then washed twice with Milli Q water. The size of AuNps prepared ranged between 5 and 30 nm, as determined by X-ray diffraction (XRD) and transmission electron microscope (TEM) (data not shown).

### 2.3. Synthesis of nano-ureases from C. ensiformis and C. cajan using AuNps and their characterization

AuNps were thoroughly rinsed with 50 mM sodium phosphate buffer, pH 7.0 and air-dried. They were activated with cysteamine under dark conditions at 25 °C for 4 h [AuNp–Cysteamine]. The sandwich cross-linker, glutaraldehyde (binds at one end to cysteamine and other to enzyme using amide linkages) was added overnight to cysteamine activated AuNps at 25 °C (AuNp–Cysteamine–Glutaraldehyde). Ureases from *C. ensiformis* and *C. cajan* (at pH 7.0) were taken for its immobilization onto AuNp–Cysteamine–Glutaraldehyde by incubating it overnight at 4 °C (AuNp–Cysteamine–Glutaraldehyde–Urease called as Nano-urease). Each step of immobilization of urease onto AuNps were followed by centrifugation at 5000 rpm for 5 min at 25 °C and subsequent washing using 50 mM sodium phosphate buffer, pH 7.0 and were air-dried. The scheme of urease (from *C. ensiformis* or *C. cajan*) immobilization onto AuNps is represented as:

AuNPs → Cysteamine → Glutaraldehyde → Urease

Morphology and size of nano-ureases were determined using transmission electron microscope (TEM; Technai 20 $G^2$, 200 kV). For TEM studies, drop of sample was put on electron microscope 200 mesh copper grid and dried by putting it under light lamp. The micro-structural details of nano-ureases were obtained using secondary electron imaging mode by scanning electron microscope (SEM; Philips: XL 20). For SEM studies, samples were sprinkled on the stub having layer of silver glue for striking the particles. Fourier transform infrared spectroscopy (NICOLET 57000, FT-IR Thermo Electron Corporation) was used to analyze the presence of various groups on nano-ureases. For FT-IR studies, samples were made by mixing the material with KBr powder followed by vacuum drying. The spectra were recorded in reflection mode using Perkin Elmer FT-IR in the range 600–4000 $cm^{-1}$ wave numbers.

### 2.4. Experimental design and statistical methodology

Preliminary experiments were performed to determine the high and low level factors for amount of AuNps (mg/mL), cysteamine (mM), glutaraldehyde (%) and amount of protein (μg). It was found to be, AuNps; (1, 10), cysteamine; (1, 10); glutaraldehyde; (1, 5) and amount of protein; (50, 200). It was found using $2^4$ designs that factors, glutaraldehyde and amount of protein play a significant role in synthesis of nano-urease (data not shown). It was also found from these experiments that 10 mg/mL of AuNps and 3 mM of cyteamine can be taken for further optimization with varying glutaraldehyde (%) and amount of protein (μg). RSM using CCD was applied to optimize the synthesis of nano-ureases from *C. ensiformis* and *C. cajan*, using significant parameters. The CCD was applied using Design Expert software (Stat Ease, 8.0.3 version). The range and the levels of the variables (amount of protein and glutaraldehyde) under investigation in this study are given in Table 1. The response, *Y* (% immobilization efficiency) was related to the selected variables by a quadratic model as follows:

$$Y = \beta_o + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{1} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j$$