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Research Article

SCREENING OF URICASE PRODUCING MICRO-ORGANISMS AND URICASE ESTIMATION: A SIMPLE AND NOVEL APPROACH

HARINATH DWIVEDI[1], KUSUM AGRAWAL[2], SHUBHINI A SARAF[1*]

[1]Department of Pharmaceutics, Faculty of Pharmacy, Babu Banarasi Das National Institute of Technology and Management, Lucknow (Uttar Pradesh), India. [2]School of Biotechnology, Shobhit University Meerut (UP), India. Email: shubhini.saraf@gmail.com

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ABSTRACT

The objective of the work was to develop a new method for screening of microbes for uricase production and estimation of uricase thereof. This was achieved by utilizing the fact that uric acid dissolves on being acted upon by uricase. The proposed method is a novel, inexpensive, simple and sensitive technique for screening and estimation of uricase.

Keywords: Uricase estimation, Enzyme estimaton, Uricase plate assay method.

INTRODUCTION

Enzymes are biocatalysts, thermo labile in nature and highly specific molecules for their action on substrate. These enzymes are synthesized by living cells only[1]. Uricase is an enzyme which can be synthesized by several wild microbes[2]. Identification of natural microbes which produce uricase is a difficult task, because it requires each microbial colony to be tested for the presence of uricase, by using estimation kits available in the market. These have a high cost and require specific critical environmental conditions for conduction of the tests. Uric acid is a weak acid which has a maximum solubility of 7 mg/dL in plasma. Normal plasma urate levels are between 3.3 to 6.9 mg/dl. Uric acid is degraded by urate oxidase (uricase) to allantoin as described previously [3,4,5]. Allantoin has a much higher solubility which is 400 mg/dl in plasma and water at 25^{0}C.

Estimation of enzyme activity in body fluids is an old concept which has often been utilized as an aid to diagnosis. Uricase is widely used to monitor serum uric acid for laboratory diagnosis of gout, kidney function and hyperuricemia-associated diseases as described previously [6, 7]. Elitek T^M is the available intravenous dosage form of uricase manufactured by Sanofi Aventis and Elitek International Corporation. Uricase can be estimated by quantifying uric acid by its distinctive absorbance at 293 nm or by quantifying hydrogen peroxide, one of the uricase products, by peroxidase-coupled analysis[8].

Enzyme purification techniques are required whenever commercial use of enzymes is made. The repeated estimation of enzymes is required at different steps of the purification process to find out the effectiveness of the process. The objective of the present study was to develop a new and simple method for identification of uricase producing microbes and subsequent estimation of the uricase.

MATERIALS AND METHODS

Quantitative estimation of uricase by enzyme assay kit:

Quantitative estimation of the produced uricase was first confirmed by Amplex Red uric acid/uricase assay kit (A22181). The Amplex Red uricase assay kit provides an ultrasensitive method for detecting uricase activity. In the assay, uricase catalyzes the conversion of uric acid to allantoin, hydrogen peroxide and carbon dioxide. The H_2O_2 in presence of horseradish peroxidase (HRP) reacts stoichiometrically with Amplex Red reagent to generate the red fluorescent oxidation product, resorufin. Resorufin gives maximum absorbance at its lambda max 560 nm.

Procedure for quantitative estimation of uricase by assay kit

Preparation of standard graph

Five dilutions of uricase were prepared (5 m Unit/ml, 10 m Unit/ml, 15 m Unit/ml, 20 m Unit/ml and 25 m Unit/ml). A volume of 50 µl of each dilution was added in separate wells of a 96 well ELISA plate. Reaction buffer of 1x provided with the kit was used to dilute the standard uricase enzyme solution. A volume of 50µl of working solution which contained amplex red reagent, HRP (Horse radish peroxidase) solution, 5 mM uric acid solution and 1x reaction buffer was added in the respective wells. The plate was incubated at 37ºC temperature for 30 minutes. After incubation the optical absorbance for the standard solutions were recorded by ELISA micro plate reader at 560 nm. Absorbance was recorded and a graph plotted between absorbance and concentration of uricase (Fig.1.).

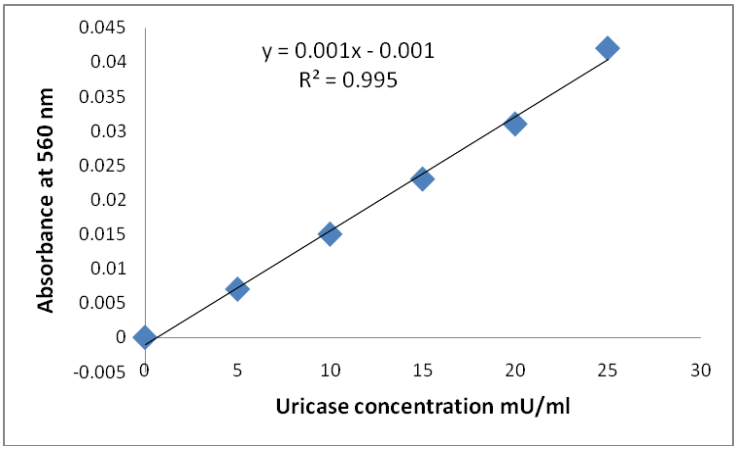


Fig. 1: Standard graph for uricase estimation

The proposed method for screening the uricase producing microbes is based on the development of the zone of clearance around the well formed in the solidified agar medium containing uric acid due to the fact that uric acid dissolves, on being acted upon by uricase.

Method for screening uricase producing microbes by the proposed method

A new method was proposed for the screening of uricase producing microbes. Microbes from a soil sample were cultured in the LB (Luria Bertani) nutrient broth medium[9]. Different microbes were isolated using spread plate method. Isolated individual colonies were grown in LB nutrient broth medium (pH 7.5) at 37^{0}C. For sub-culturing, the isolated microbes at a concentration of 5.0 %v/v of inoculum, at the stage of 0.8 optical density and 600 nm of wavelength of light , were inoculated into already sterilized 25ml LB nutrient broth medium (pH 7.5) in a conical flask. The inoculated nutrient broth was incubated at 37^{0}C for 48 hours. After the growth of the microbes at the end of incubation period, 50 µl of microbial culture was filled in a well, formed with the help of a cork borer in a solidified 2.0 % w/v agar medium in water, containing 0.5 % w/v uric acid, inside a petriplate. Sterilized nutrient broth medium was used as a control in a separate well. The plate was incubated at 37^{0}C for a duration of 24 hours. The term *Zone of Clearance* was coined for the clear zone developed around the well formed in the solidified medium (Fig.2.). This method was henceforth referred to as the uricase plate assay method. Zone of clearance developed around the wells for each microbial sample was measured and thus the production of uricase enzyme by the microbe was established. The production of uricase enzyme by the microbes was also confirmed by quantitative estimation performed with the help of Amplex Red Uric acid/Uricase assay kit (A22181) procured from Molecular Probes Invitrogen Detection Technologies, USA.

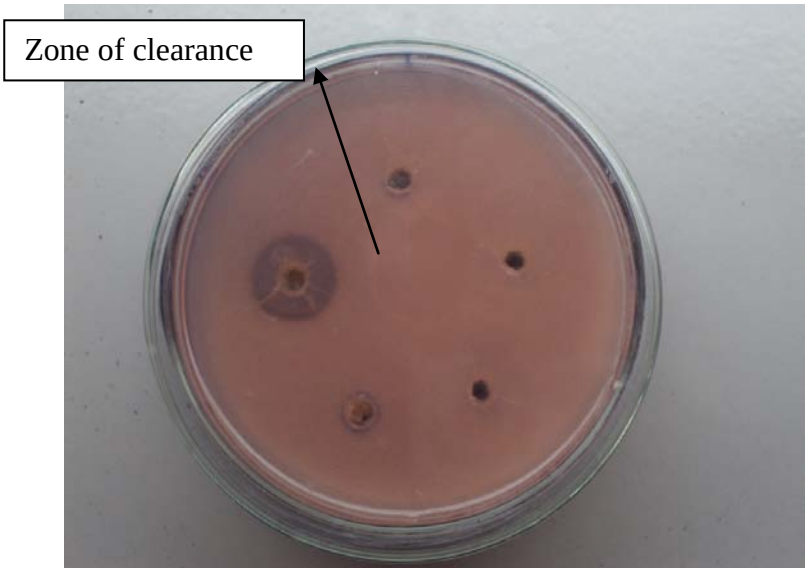


Fig. 2: Zone of clearance developed around the well indicating the presence of microbes capable of producing uricase.

Quantitative estimation of uricase by the proposed method

Procedure for uricase plate assay method

A 25 ml agar solution (2% w/v) was prepared in distilled water with the aid of slow heat. The solution was boiled to get a clear solution. The solution was allowed to cool up to about 45 ºC. At this temperature, when the agar solution remains in a liquid condition, 0.5% w/v uric acid and 0.5%w/v of safranine dye was added to it with proper mixing.

When four different samples of uricase having their concentration 100, 50, 25 and 12.5 mU/ml were estimated for the amount of uricase by the proposed uricase plate assay method, the diameter of zone of clearance developed were found to be the directly proportional to the logarithmic concentration of the uricase added to the well.

RESULTS AND DISCUSSION

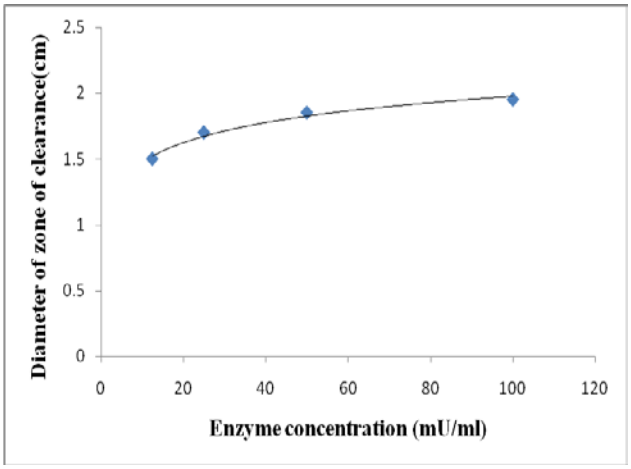
An attempt was made to correlate the fact that uric acid has a solubility of 7 mg/dL in water while allantoin is more soluble than uric acid having a solubility of 400 mg/dL and that allantoin is formed when uricase acts upon uric acid[3]. Utilizing this as the basis, a novel, simple and inexpensive method for estimation of uricase was developed. In the method, a sample containing uricase when added to a well formed in the solidified agar converts the suspended uric acid into clear and water soluble product allantoin, thereby developing a clear zone around the well. Uric acid is known to degrade above 60^0 C, thus care was taken to add it only after the medium cooled down to 45^0 C. The samples which do not have uricase, fail to develop a zone of clearance. The diameter of the zone of clearance developed was found to be directly proportional to the logarithmic concentration of uricase. Addition of a small amount of safranine dye in the medium in which wells are formed, gives a better contrast between zone of clearance developed and rest of the medium resulting in better visualization.

$$\text{Uric acid} + \text{Uricase} \longrightarrow \text{Allantoin} + H_2O_2 + CO_2$$

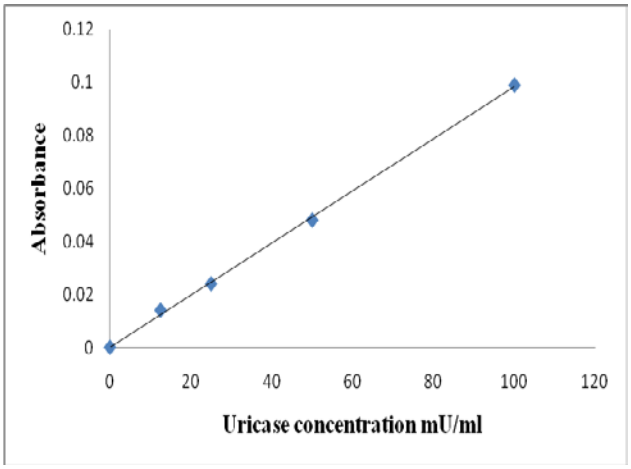
When samples of uricase having different concentration were estimated for the amount of uricase, by the proposed uricase plate assay method, the diameter of zone of clearance developed was found to be directly proportional to the logarithmic concentration of uricase present in it. (Fig.3.).

The proposed method, being inexpensive and simple, can be used for screening of microbes producing uricase, quantitative estimation of uricase, as well as to demonstrate the same in the laboratory. The proposed method for estimation of uricase was found to be effective and the results were corroborated by the readings obtained with the uricase assay kit (Amplex Red uric acid/uricase assay kit - A22181 by Molecular Probes Invitrogen Detection Technologies USA). When compared with other methods as described previously [10, 11, 12], it was found to be sensitive to detection and estimation of uricase

concentration for a minimum of 0.5 mU/ml and higher concentrations.



(a)



(b)

Fig. 3(a): Graph indicating the relationship between the enzyme concentration and diameter of the zone of clearance developed. Fig. 3(b): Graph indicating the relationship between the enzyme concentration and optical absorbance at 560 nm wavelength of light.

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