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Novel, Effective and Sensitive Plate Assay Method for Detection and Screening of Urease Producing Microorganisms

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A B S T R A C T

Urease is a kind of amidohydrolase produced by ureolytic microorganisms. Microbial ureases have significant application in biotechnology and are of clinical relevance. The urease producing microorganisms are conventionally screened on urea containing phenol red plates. The contrast of the zone obtained due to change in pH from light orange to pink is not very sharp and distinct. Utilization of proteins by urease producing microorganisms and prolonged incubation also increases the pH of medium resulting in false positives. In the present investigation a novel, rapid, efficient and sensitive plate assay was developed for detection and screening of urease producing microorganisms. Bacteria producing ureases were screened on nutrient urea agar whereas fungi were screened on potato dextrose urea agar medium. The plates used for detection were incubated at respective temperatures and flooded with Gram's iodine solution. Clear zones were formed around the colonies producing urease, by which the enzyme producers and non producers could be differentiated. The present method is more accurate and suitable for screening of both bacteria and fungi producing extracellular urease. Futhermore, potent isolates can be easily detected based on the diameter of hydrolyzed zone formed.

Introduction

Urease (EC 3.5.1.5), a nickel-dependent metalloenzyme is the first enzyme to be purified and crystallized (Sumner, 1926). The enzyme catalyzes the conversion of urea to ammonia and carbamate. The compound spontaneously hydrolyzes at physiological pH to generate carbonic acid and a further ammonia molecule (Andrews *et al*., 1984). Several reports appeared in the literature on urease producing microorganisms (Cui *et al*., 2022; Mekonnen *et al*., 2021; Krajewska *et al*., 2012). The presence of ureolytic activity as a virulence factor has been well studied from a clinical prespective in microorganisms which have a significant function in chronic diseases like gastric ulceration, urinary stones, urolithiasis, pyelonephritis, atherosclerosis and rheumatoid arthritis (Konieczna *et al*., 2012;

Arabski *et al*., 2010; Nicolle, 2002; Samtoy and DeBeukelaer, 1980).

Microbial ureases have a wide range of applications in biotechnology, agricultural, medicinal and engineering fields. The most extensive analytical application of urease has been for the quantification of urea in blood and urine (Francis *et al*., 2002). Quantitative determination of urea is important in environmental monitoring. Urease is used in the development of urea biosensors (Jakhar and Pundir, 2018). The enzyme is used for bioremediation in the treatment of urea rich industrial fertilizer effluents by immobilizing crude urease onto polyester which is having high flow through property in columns (George *et al*., 1997). There has been a growing demand for application of urease in food science (Krajewska, 2009).

However, the urease produced by microbes is mostly intracellular with low yield and difficult to extract and purify the enzyme. The enzymes produced are often insufficient for therapeutic and other industrial applications. Thus, there is an urgent need to develop methods for rapid screening of microbes which produce this enzyme extracellularly and in high yields.

The earlier phenol red plate assay method was developed for screening of extracellular urease producing bacteria from urea agar medium. Ammonia produced from hydrolysis of urea alkalinizes the medium and the change in pH is detected by a gradual transition of colour from light orange at pH 6.8 to pink at pH 8.1 (Christensen, 1946). Prolonged incubation causes alkaline reaction in the medium which is not due to urease activity. Utilization of proteins by urease producing microorganisms increases the pH of the medium resulting in false positives. In the present investigation a novel, sensitive, efficient and contrasting rapid plate assay method is reported for the screening of extracellular urease producing microbes using Gram's iodine and quantitative estimation of urease activity of the identified isolates by submerged fermentation.

Materials and Methods

Soil collection

Urea rich soil samples were collected from paddy field crops located in Chittoor, Andhra Pradesh, India.

Isolation of microorganisms from soil by the serial dilution-agar plate method

From each plot the samples were taken at random to make a composite sample. The samples of soil were brought to the laboratory by collecting in sterile test tubes. The soil samples were serially diluted to tenfold dilution series with 0.85 % sodium chloride. One ml aliquot of 10^{-7} dilution was used for each agar plating method.

Chemicals

Iodine and potassium iodide were purchased from Sigma-Aldrich (India). Nessler's reagent and other chemicals used were of analytical grade from Hi-Media (India).

Preparation of media for screening of strains

The newly developed nutrient urea agar (NUA) medium for screening of bacteria has the following ingredients (gm/L): peptone, 5.0; beef extract, 3.0; sodium chloride, 5.0; urea, 20.0 and agar, 20.0. All the ingredients except urea were autoclaved in 250 ml Erlenmeyer flasks containing 90 ml of the medium. The pH of 2 % urea solution was adjusted to 7.0 and 10 ml quantities of urea solution were filter sterilized and added to the 90 ml of sterilized nutrient medium which was maintained at 40 °C-50 °C. Potato dextrose urea agar (PDUA) medium was prepared for fungal screening, containing the following ingredients (gm/L): starch (potato), 4.0; dextrose, 20.0; urea, 20.0 and agar, 20.0. The sterilization procedure of the medium described in earlier for the screening of bacteria was repeated for screening of fungi with PDUA medium. Streptomycin sulphate at concentration of

30 mg/1000 ml was added to the prepared PDUA medium.

Gram's iodine solution was prepared by dissolving the following ingredients (gm/300 ml of distilled water): potassium iodide, 2.0 and iodine, 1.0.

NUA plates were inoculated with aliquot cultures and incubated at 37 °C for 24 hr where as inoculated PDUA plates were incubated at 25 °C for 72 hr. Uninoculated plates were used as control.

Culture preparation

One of the 10^{-7} dilution plates with unselected isolates was used to check the quantitative estimation of hydrolyzed zone with Gram's iodine. From the remaining plates of the designed medium a total of 120 bacterial and 90 fungal strains were isolated randomly. The selected promising bacterial isolates were identified as *Bacillus subtilis* and *Bacillus licheniformis* while the fungal isolate was identified as *Aspergillus niger* on the basis of 16S rRNA gene and gene sequence analysis with Midilabs.

The identified isolates were labeled as IICT-PN-1 (*Bacillus subtilis*), IICT-PN-2 (*Bacillus licheniformis*) and IICT-PN-3 (*Aspergillus niger*). Nutrient agar was used for maintaining bacterial cultures and potato dextrose media for fungal cultures. The identified isolates were subjected for further studies. These isolates were subcultured on NUA medium and PDUA medium and preserved for testing in refrigerator. Pure cultures of the selected isolates obtained from streak-plate method were flooded with Gram's iodine for the detection of hydrolyzed zones. Two best isolates were selected.

Evaluation of urease activity

The nutrient urea broth was used as production medium for quantitative estimation of bacterial urease and potato dextrose urea was used for fungal urease.

Urease production was carried out in Erlenmeyer flasks incubated on rotary shaker at 220 rpm, 30 °C at pH 7.0. Uninoculated culture media served as controls. Five ml quantities of the samples were withdrawn at an interval of every 6 hr and checked for enzyme activity as per the Nessler's reagent method (Das and Kayastha, 1998) recording the absorbance at 405 nm in an Agilent Cary spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberated one micromole of ammonia from urea per minute under standard assay conditions.

Results and Discussion

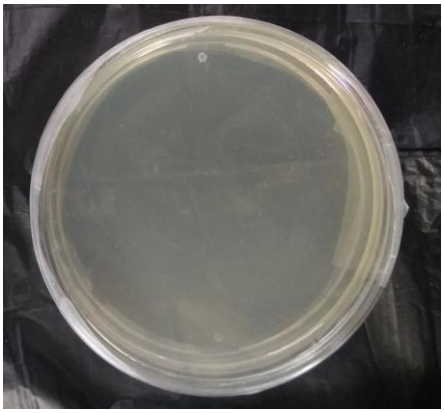
Several investigations have highlighted the potential uses of ureases. In recent years the production of bacterial urease has gained importance. The primary objective of this research was to isolate and identify the strains producing the highest yield of urease with newly designed NUA and PDUA medium with Gram's iodine solution and identifying the urease activity of selected isolates. Collected soil samples were used for the isolation of urease activity exhibiting microorganisms employing the serial dilution technique. After 24 hr of bacterial and 72 hr of fungal incubation, clear zones were developed around the colonies flooded with Gram's iodine solution indicating urease production capabilities of the bacterial (Fig. 1) and fungal strains (Fig. 2).

One ml aliquots of 10^{-7} dilutions used for pour-plate method showed growth of bacterial colonies in NUA medium (Fig. 1 c) and fungi colonies in PDUA medium (Fig. 2 c). Uninoculated plates were used as control for bacteria (Fig. 1 a) and fungi (Fig. 2 a).

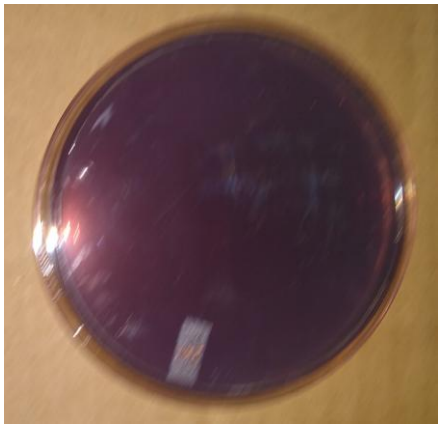
Two best isolates were selected from 120 isolates of urease producing bacteria and were identified as *Bacillus subtilis* and *Bacillus licheniformis*. Streak-plate method was carried out in NUA medium with the identified isolates *Bacillus subtilis* (Fig. 1 e) and *Bacillus licheniformis* (Fig. 1 g). On incubation the plates were flooded with Gram's iodine solution.

Fig. 1 Detection of urease producing bacteria on NUA plates flooded with Gram's iodine

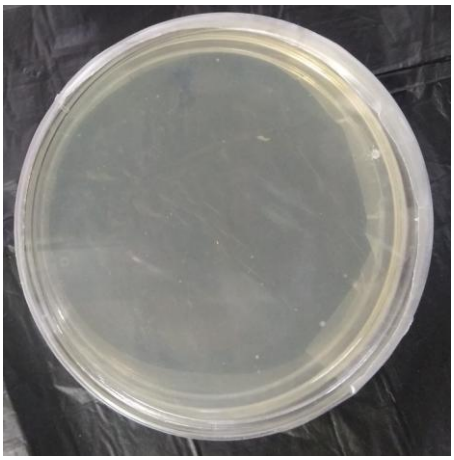
a. Control



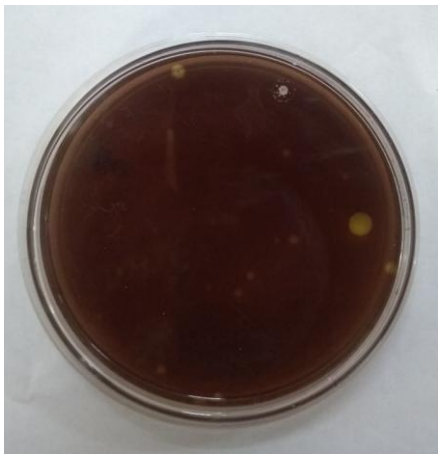
b. Control plate flooded with Gram's iodine



c. Pour-plate method of bacteria in NUA medium

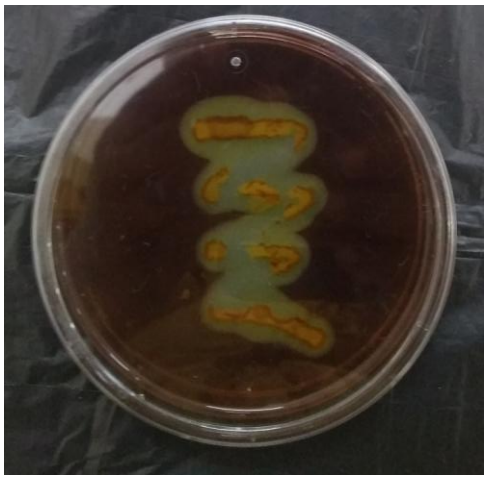


d. Formation of clear hydrolyzed zones with unselected strains in NUA medium of bacterial pour-plate method flooded with Gram's iodine solution

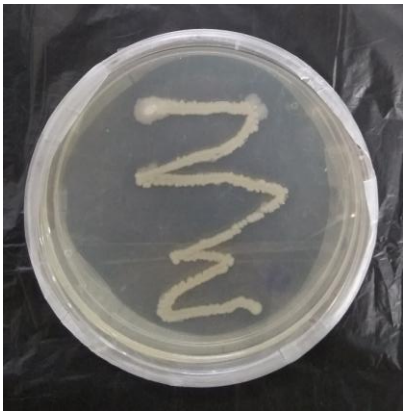


e. Streak-plate method in NUA medium with *Bacillus subtilis* (IICT-PN-1)

f. Urease activity of *Bacillus subtilis* (IICT-PN-1) in NUA medium flooded with Gram's iodine solution



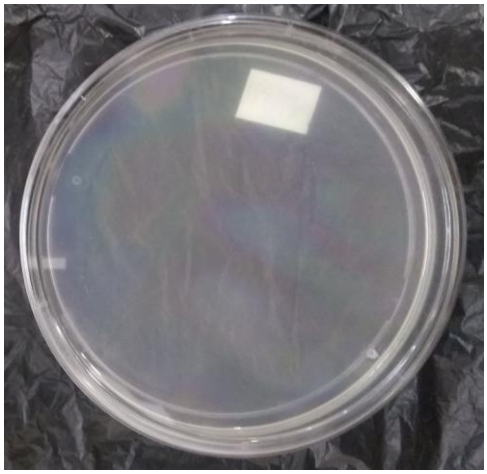
g. Streak-plate method in NUA medium with *Bacillus licheniformis* (IICT-PN-2)



h. Urease activity of *Bacillus licheniformis* (IICT-PN-2) in NUA medium flooded with Gram's iodine solution

Fig. 2 Detection of urease producing fungi on PDUA plates flooded with Gram's iodine

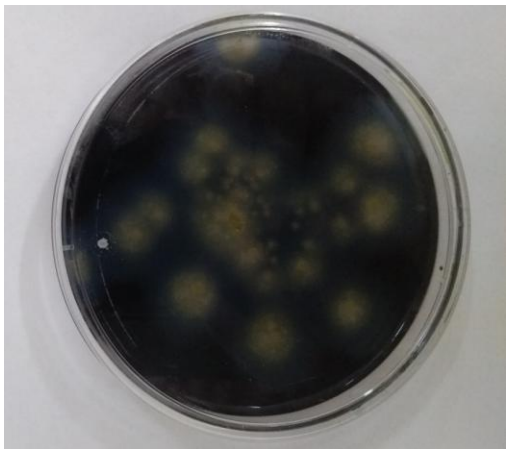
a. Control



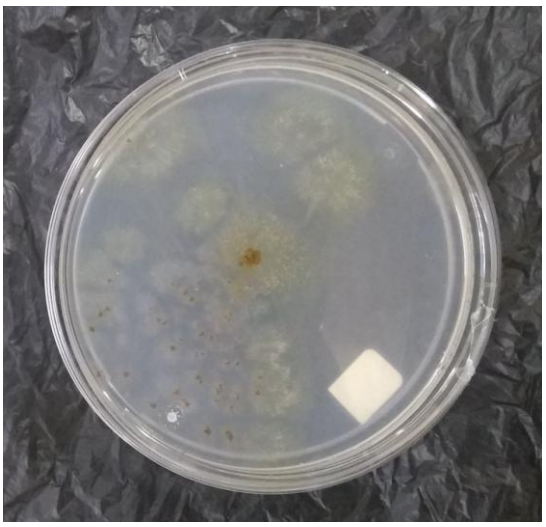
b. Control plate flooded with Gram's iodine

c. Pour-plate method of fungi in PDUA medium

d. Formation of clear hydrolyzed zones with unselected fungal strains in PDUA medium by pour-plate method flooded with Gram's iodine solution



e. *Aspergillus niger* (IICT-PN-3) inoculated in PDUA medium



f. Urease activity of *Aspergillus niger* (IICT-PN-3) in PDUA medium flooded with Gram's iodine solution

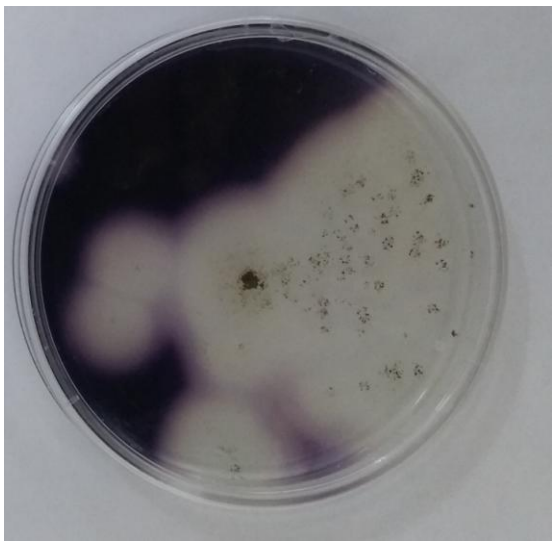
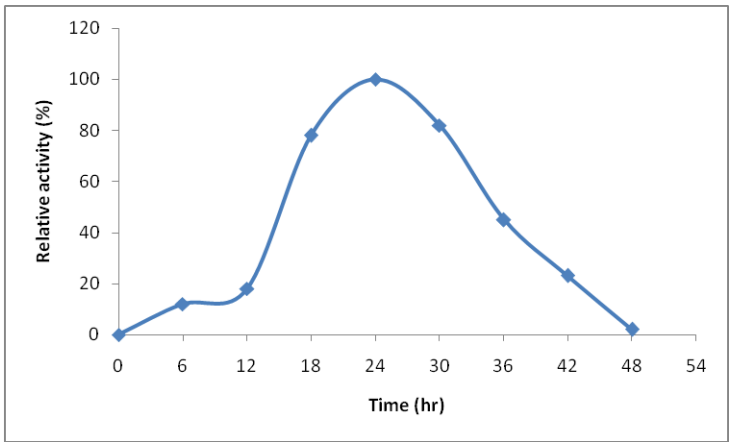
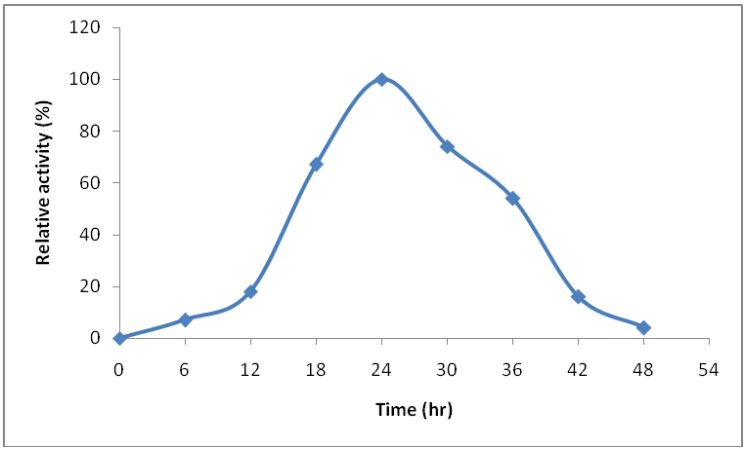


Fig. 3 Urease production of the identified isolates

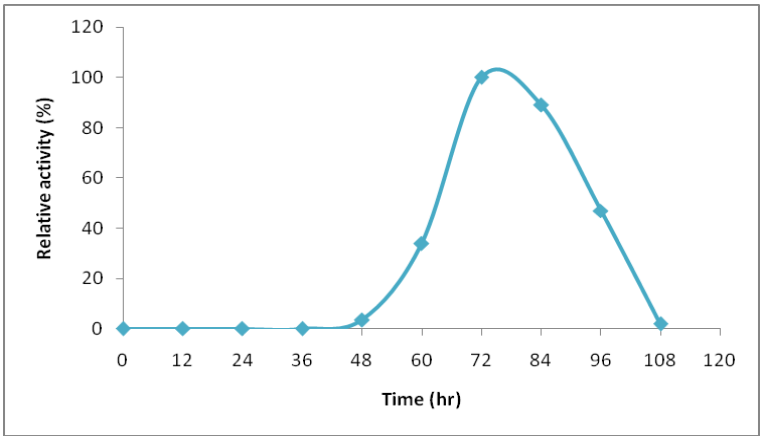
a. *Bacillus subtilis* (IICT-PN-1)



b. Bacillus licheniformis (IICT-PN-2)



c. Aspergillus niger (IICT-PN-3)



Clear hydrolyzed zones around the colonies were developed in pour-plate (Fig. 1 d), *Bacillus subtilis* (Fig. 1 f) and *Bacillus licheniformis* (Fig. 1 h). Uninoculated control plate flooded with Gram's iodine solution was completely stained (Fig. 1 b).

Pour-plate method was utilized to isolate *Aspergillus niger* strain producing higher yields of urease enzyme. The isolate was inoculated on PDUA plate (Fig. 2 e). Fungal growth plates were flooded with Gram's iodine solution showed clear hydrolyzed zones around the colonies in pour-plate method (Fig. 2 d) and plates inoculated with *Aspergillus niger* (Fig. 2 f). Uninoculated PDUA control plates stained with Gram's iodine solution were completely stained (Fig. 2 b). Hydrolyzed zones of 5.7 mm and 5.2 mm were observed with *Bacillus subtilis* and *Bacillus licheniformis* and 9.3 mm for *Aspergillus niger*. A sharp, distinct, clear and prominent zone of enzyme hydrolysis was observed in the plates inoculated with the identified isolates. The newly developed method strongly indicates that the developed novel method can be effectively employed for screening of urease producing bacteria on NUA medium and fungal ureases on PDUA medium with Gram's iodine solution.

Urea solubilization efficiency

The effective urea solubilizers were evaluated for their efficiency to produce extracellular urease. The ability of microbes to dissolve urea can be determined by analyzing the ammonia liberated in the culture medium by Nessler's method (Das and Kayastha, 1998) at 405 nm. The isolate *Bacillus subtilis* which is the predominant bacteria involved in enhanced urease production of 214 U/ml. Other selected *Bacillus licheniformis* exhibited urease activity of 197 U/ml.

A higher amount of urease activity was detected with *Aspergillus niger* at 235 U/ml. Enzyme production of the identified isolates were graphically represented (Fig. 3). The results indicated that all the selected strains were able to produce urease under *in vitro* conditions.

The developed Gram's iodine method gives a sharp colour contrast between the hydrolyzed and unhydrolyzed zones of urea as compared with the zones exhibited by phenol red method. Change in colour of medium due to phenol red (Christensen, 1946) does not differentiate between two isolates exhibiting almost similar activity. The Gram's iodine urease plate assay has an advantage over the phenol red assay as it can give a clear zone of hydrolysis around each isolate instead of pH dependent change in colour of medium. The described screening procedure can be used for plate assay of isolates producing ureases. This suggests that the newly developed method can be employed for the qualitative evaluation of urease activity. In conclusion, the developed novel method clearly exhibits the clarity of the zone of hydrolysis, making the process easy, effective and rapid for the screening of large number of microorganisms producing ureases.

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References

Andrews, R. K., Blakeley, R. L., Zerner, B. 1984. Urea and urease. *Adv. Inorg. Biochem.* 6, 245–283.

Arabski, M., Konieczna, I., Sołowiej, D., Rogoń, A., Kolesińska, B., Kamiński, Z., Kaca, W. 2010. Are anti-*H. pylori* urease antibodies involved in atherosclerotic disease? *Clin. Biochem.* 43, 115–123.

Christensen, W. B. 1946. Urea decomposition as a

means of differentiating *Proteus* and *Paracolon* cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol.* 52, 461–466.

Cui, M-J., Teng, A., Chu, J., Cao, B. 2022. A quantitative, high-throughput urease activity assay for comparison and rapid screening of ureolytic bacteria. *Environ. Res.* 208, 112738.

Das, N. and Kayastha, A. M. 1998. Immobilization of urease from pigeonpea (*Cajanus cajan* L.) in flannel cloth using polyethyleneimine. *World J. Microbiol. Biotechnol.* 14, 927–929.

Francis, P. S., Lewis, S. W., Lim, K. F. 2002. Analytical methodology for the determination of urea: current practice and future trends. *Tren. Analy. Chem.* 21, 389–400.

George, S., Chellapandian, M., Sivasankar, B., Jayaraman, K. 1997. A new process for the treatment of fertilizer effluent using immobilized urease. *Bioproc. Biosyst. Eng.* 16, 83–85.

Jakhar, S. and Pundir, C. S. 2018. Preparation, characterization and application of urease nanoparticles for construction of an improved potentiometric urea biosensor. *Biosens. Bioelectron.* 100, 242–250.

Konieczna, I., Kwinkowski, M., Kolesińska, B., Kamiński, Z., Zarnowiec, P., Kaca, W. 2012. Detection of antibodies against synthetic peptides mimicking ureases fragments in sera of rheumatoid arthritis patients. *Protein Pept. Lett.* 19, 1149–1154.

Krajewska, B. 2009. Ureases I. Functional, catalytic and kinetic properties: a review. *J. Mol. Catal. B. Enzym.* 59, 9–21.

Krajewska, B., van Eldik, R., Brindell, M. 2012. Temperature- and pressure-dependent stopped-flow kinetic studies of jack bean urease. Implications for the catalytic mechanism. *J. Biol. Inorg. Chem.* 17, 1123–1134.

Mekonnen, E., Kebede, A., Nigussie, A., Kebede, G., Tafesse, M. 2021. Isolation and characterization of urease-producing soil bacteria. *Int. J. Microbiol.* 6, 1–11.

Nicolle, L. E. 2002. Epidemiology of urinary tract infections. *Clin. Microb. News* 24, 135–40.

Samtoy, B. and DeBeukelaer, M. M. 1980. Ammonia encephalopathy secondary to urinary tract infection with *Proteus mirabilis*. *Pediatrics* 65, 294–297.

Sumner, J. B. 1926. The isolation and crystallization of the enzyme urease. Preliminary paper. *J. Biol. Chem.* 69, 435–441.

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