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Review Article

A review on distribution, properties, genetic organization, immobilisation and applications of urease

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Abstract
Urease, a nickel-containing metalloenzyme is getting remarkable attention due to a diverse range of applications for mankind. Urease plays a magnificent role in various field like agriculture, analytical, geological phenomena, beverage industry and is an important diagnostic tool. Urease is mainly present in bacteria, fungi, plants and invertebrates and its manifestation in specific genera may open new vistas for its taxonomic position. Various qualitative and quantitative assays are also reported for the estimation of urease enzyme. Urease based biosensors utilizing green synthesis on nanoparticles are also trending. Recently developed inhibitors against urease were discussed in the review. Inhibitory mechanisms involving the structural similarity of the substrate through modification or derivatization can also help in rational drug design by two possible competitive ways either by mimicking monodentate urea binding or binding as a tetrahedral intermediate. Immobilisation of urease through gel entrapment, using non-covalent and covalent protein tags, cross linkage, covalent bonding, using composite films, Teflon, co-precipitation and coating on nanoparticles is also reported. This review also comprised of various application of urease including enhancement of fertility in the soil, cell to cell organization, protection to predators, treatment of various bladder related diseases and infections, analysis of urea and heavy metal ions, biocementation, pollution control by bioleaching of heavy metals and making beverages urea and ethyl carbamate free. As researchers have a keen interest in urease enzyme at present, most of its aspects were incorporated in the article to make it helpful to the scientific community for further research related to the development of new inhibitors and add on applications of urease for the upliftment of the human as well as environment.

Keywords: Applications, Enzyme Inhibitors, Genetic Organization, Immobilization, Urease

INTRODUCTION

The ureases are getting considerable attention due to their various physiological effects on mankind with diverse applications. Ureases are metalloenzymes with nickel in their active sites and belong to super family of amidohydrolases and phosphotriesterases (Saeed *et al*., 2017). Van Tiehem, in 1864, first isolated ureolytic microorganism (*Micrococcus ureae*) and Musculus, in 1874, isolated the first enzyme having ureolytic activity from putrid urine. The name “urease” was given by Miquel in 1890, whereas James Sumner, in 1926, crystallized it from Jackbean (*Canavalis ensiformis*) seeds and got Nobel prize for this in 1946 (Kappaun *et al*., 2018). Though urease was the first enzyme to crystallize, it was only in 1995 that Jabri *et al.* first reported the full 3-D structure of urease of *Klebsiella aerogenes* (Kataria and Khatkar, 2019). It took nearly 84 years to

generate 3D structure of Jackbean Urease (Abbas *et al*., 2019).

Urea acts as a nitrogen resource for many pathogenic microbes. These microorganisms utilize urea with the help of urease which breaks urea into carbamic acid and ammonia and converts carbamic acid to carbonic acid to produce more ammonia molecules (Ansari and Yamaoka, 2017).

$$CH_4N_2O+H_2O \xrightarrow{\text{urease}} NH_3+CH_3NO_2 \quad \text{Eq.1}$$

$$CH_3NO_2+H_2O \longrightarrow NH_3+H_2CO_3 \quad \text{Eq.2}$$

Under physiological conditions, the carbonic acid proton dissociates and ammonium ions cause pH to rise and interfere with host function (Rutherford, 2014).

There are two isoenzymes of urease (tissue-ubiquitous and embryo specific) encoded by two separate genes and regulatory proteins determined by unlinked genes recognized in soybean. Embryo-specific urease is abundantly present in plant species like soybean, Arabidopsis and jackbean (Sujoy and Aparna, 2013) and tissue specific urease is present in minor amount in the vegetative phase of plants (Nhung *et al*., 2019). Breakdown of amino acids in humans generates urea evenly distributed throughout the body, including epithelial lining fluids, subcutaneous adipose tissue, central nervous system and blood serum (Wernimont *et al*., 2020). Intracellular urease increases the periplasmic pH and allows protein synthesis at low pH by increasing the membrane potential. The ammonia released during the process provides acid neutralizing as well as acid buffering capabilities which makes microorganisms to increase pH of their periplasm and microenvironment (Maier and Benoit, 2019). Due to the widespread availability of urease in nature, it plays a significant role in agriculture, animal health, beverages industries, geological phenomena, waste water reclamation and as an important diagnostic tool. Infection by bacteria having urease may cause stone development which protects the pathogen by surrounding it (Odoemene and Adiri, 2019).

SOURCES OF UREASE

Urease enzyme is mainly found in bacteria (archaebacteria also), fungi, plants and invertebrates and its limited occurrence in certain genera prove helpful for taxonomic assignment. It can be present membrane-bound, inside the cytoplasm or as a free form in the extracellular matrix. 17-30 % of the cultivable bacterial population from soil contains urease (Cheng *et al*., 2017) and urease plays a significant role in nitrogen metabolism and increases nitrogen availability for the plants. Numerous strains of microorganisms like *Peptostreptococcus productus, Ruminococcus bromii*, *Succinivibrio dextrinosolvens, Prevotella ruminicola (Bacteroides ruminicola*), *Bifidobacterium*, *Treponema*, *Butyrivibrio* species had been reported for urease activity in ruminants (Patra and Aschenbach, 2018). In high urea diet ruminants, the elevated urease activity leads to ammonia/urea toxicity. Higher concentrations of ammonia in gastrointestinal tract of non-ruminants mainly damaged gastrointestinal mucosa resulting in the impairment of nutrient absorption and thus retarded the growth of the animal (Patra and Aschenbach, 2018). Non ruminant animals like pigs, rats, mice, cat, rabbits and human also contain microbes like *Peptococcus magnus, Clostridium innocuum*, *Clostridium coccoides*, *Peptostreptococcus micros*, , *Fusobacterium russii*, *Fusobacterium* sp., *Streptococcus* sp., *Mitsuokella* (Bacteroides) *multiacidus*, *Eubacterium limosus*, *Staphylococcus* sp., *Selenomonas ruminantium* which perform substantial urease activity (Wu, 2022).

Many strains of microbes like *Proteus mirabilis*, *Staphylococcus saprophyticus*, *Campylobacter pyloridis, Helicobacter pylori*, *Clostridium perfringens*, *Proteus*, *Klebsiella* species (Rutherford, 2014), *Salmonella* sp., *Staphylococcus aureus*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Ureaplasma urealyticum* in humans cause various diseases like urinary catheter encrustation, hepatic coma, hepatic encephalopathy, urolithiasis, pyelonephritis, Parkinson's disease and gastritis (Kataria and Khatkar, 2019).

Urease activity is also found in invertebrates like *Aplysia californica* (Carey *et al*., 2016) and Land snail *Otala lactea* (Liu *et al*., 2021a). Ammonia produced due to urease activity acts as proton acceptor enhanced the biological deposition of the Calcium carbonate (Liu *et al*., 2021b).

Many Fungal species like *Coccidioides immitis* (Javadi *et al*., 2018), *Rhodotorula* spp. and *Cryptococcus neoformans* infect humans lungs and damage human immune system in a life-threatening way (Yockey *et al*., 2019). *Aspergillus niger* (Khan *et al*., 2019), *Schizosaccharomyces pombe* (Kataria and Khatkar, 2019) and *Aspergillus nidulans* (Khan *et al*., 2019) showed significant urease activity in a purine degradation manner (Toplis *et al*., 2020).

The best-characterized plant urease is that from Jack bean (*Canavalia ensiformis*) (Saem *et al*., 2015), *Arabidopsis*, soybean (*Glycine max*) (Wiebke-Strohm *et al*., 2016), mulberry (*Morus alba*) (Kumar, 2017), *Cajanus cajan*, *Pisum sativum* (Bedan, 2020), chickpeas (*Cicer arietinum*) (Olenska *et al*., 2020) and cotton (*Gossypium hirsutum*) seeds (Mahmud *et al*., 2019). It is abundantly present in seeds of many plant families, like seed protein in many members of the Leguminosae, Pinaceae, Asteraceae and Cucurbitaceae, and (Dalton, 2018) and vegetative tissue in lower plants (Nhung *et* al., 2019).

UREASE ASSAY

Different assays for urea detection are used according

to their interference susceptibility, sensitivity to inhibitors and ease of use. Both qualitative and quantitative approaches are used for different purposes. Change in colour due to pH change by ammonium ions released after bacterial urease reaction can be detected on a microbiological medium with phenol and urea as a pH indicator but this method is restricted to the multiplication of bacteria (Chang *et al*., 2017). NADH dependent glutamate dehydrogenase also use ammonia released after urease activity and can be measured spectrophotometrically and specific electrodes are also available for detection and monitoring of ammonia release (Dilrukshi and Kawasaki, 2016). Gel electrophoresis method is also used in which sample with urease is electrophoresed on acrylamide and agarose gel and active protein can be detected after incubation of gel in solution of phenol red and urea and this assay can help in protein size estimation (Nam *et al*., 2016).

Some common quantitative assays like phenol-hypochlorite assays, in which ammonia reacts with phenol hypochlorite and results in the formation of indophenol which can be easily measured spectrophotometrically and even a small amount of ammonia is detectable by this process, but the samples prepared were mainly sensitive to temperature, pH buffer and inhibitors (Hashihama *et al*., 2015). Ammonia reactions with Nessler reagent in pH indicator solution after dilution with HCL help to determine the amount of ammonia by spectrophotometric method (Dong *et al*., 2017). Direct monitoring of ammonia is also possible by using an ion-selective electrode by making the ionic strength constant (Toth *et al*., 2018). Carbon isotopes like C^{14} and C^{13} labelling of urea can be monitored by scintillation counter or mass spectrometer and are mainly used for diagnosing *H. pylori* (Braissant *et al*., 2020). The scheme of the different assays for urease detection is shown diagrammatically in Fig.1.

UREASE DETECTION TESTS

Christensen's urea agar

This Christensen urea (Christensen, 1946, Duran Ramirez *et al*., 2022) was developed for the differentiation of *bacilli* in 1946 and mainly determined the urea splitting property of organisms with the production of urease. Streaking of the entire slant surface was done with a heavy inoculum of 18-24 hrs pure culture, the butt was served as a colour control and incubation of tubes was done at 35^0C with loose caps. The colour change was monitored after an interval of 6 hours, 24 hours and every day for 6 days. The observation of pink (fuchsia) colour and even small detection of colour is considered for the presence of urease positively and prolonged incubation can give false results. Some rapid urease positive organisms like *Protease* (*Morganella morganii*, *Proteus* sp., and *Providencia stuartii* strains) can produce a strong positive reaction within 6 hours of incubation, slow positive organisms (*Enterobacter* or *Klebsiella* sp*)* mainly produce weak positive results after 6 hours of incubation which will spread and become intensified on further incubation till 6 days and persistent yellow colour indicates the urease negative organism.

Urease detection using polydiacetylene vesicles

One more colorimetric urease assay using polydiacetylene vesicles (Jannaha and Kima, 2019) has been reported. Ammonia produced after urease action increased the pH, resulting in the colour change of polydiacetylene vesicles from blue to red. This detection method is sensitive, simple, rapid and economical.

PROPERTIES OF UREASE

The urease enzyme catalyses the urea hydrolysis reaction and increases the rate of reaction by approximately 1014 times. Several biochemical and kinetic studies are reported to determine the chemical nature and properties of the enzyme. Urease enzymes can be found extracellularly, bound to the membrane or in the cytoplasm. The urease extracted from jack-bean (*Canavalia ensiformis*) is best characterized. In general, the molecular weight of fungal urease ranges from 180-540 kDa, bacterial urease from 200 kDa to1.06 MDa and that of plant urease range from 175-540 kDa (Kappaun *et al.*, 2018). Trimers α_3 or hexamers α_6 of identical subunits of 90 kDa are assembled to form the structure of plant and fungal urease (Carlini and Ligabue-Braun, 2016). The bacterial urease mainly comprises three different subunits, one large α subunit of 60–76 kDa and the remaining two are small β of 8–21 kDa and γ of 6–14 kDa forming a formula of $(\alpha\beta\gamma)_3$ (Kappaun *et al.*, 2018).

Molecular structure of Jack bean urease comprises forty-seven -SH groups and among them 4 to 8 of these groups are essential for its activity as it hydrolyses the non-polar C-N bond in amide (Kataria and Khatkar, 2019). The optimum urease enzyme temperature and pH mainly lie between 35^oC to 70^oC (Kumari and Rao, 2017) and 6.0 to 7.0, respectively (Baltas, 2017). The maximum activity of jack bean urease was reported at 65^oC and remains inactive above 70^oC (Dilrukshia and Kawasakib, 2016). The other plants have optimum pH range from 5.25 to 8.75, bacterial urease is 4.5 to 10.5 and fungal urease is mainly active in alkaline medium at pH above 8.0. (Kappaun *et al.*, 2018). *Asparagus* urease has optimum activity at 35^oC and pH 7.0 (Zusfahair *et al.*, 2018). Isoelectric point for plant urease is 5.1. Ureases from fungal sources also have equivalent isoelectric point with little fluctuations and 4.3-6.0 range of isoelectric point is followed by ureases from bacterial sources (Kappaun *et al.*, 2018).

Purified urease exhibited simple Michaelis-Menton kinetics and narrow substrate specificity (Navanietha Krishnaraj *et al*., 2017) and Km value for fungal, plant and bacterial urease range from 1.03-4.1 mM, 0.2-3.0 mM and 0.2-32 mM respectively for urea (Kappaun *et al*., 2018). The free energy of activation ΔG_a of immobilised urease is greater than the free urease (Al-Ansari *et al*., 2019). Urease enzyme with high activity can be maintained/ stored at 4°C for almost a month without significant loss of activity whereas Low-activity urease solutions should be stored at 4°C for only one day (Alev *et al*., 2019). Some of the characteristics of urease are summarized in Table 1.

STRUCTURE AND GENETIC ORGANIZATION OF UREASE

The amino acid sequence of prokaryotic and eukaryotic urease shows co-linearity in smaller subunit in the corresponding region. Different urease shows almost 55% identical in their gene sequence, proving their divergence from a common ancestor. This is a nickel-containing enzyme that mainly requires some additional proteins for their hydrolytic activity. So, the activity requires genes coding for structural polypeptide and accessary proteins should be located in joint clusters (Armbruster *et al*., 2018). Bacterial urease has several biological effects, so their structure is also important for understanding urease's genetic organization and active mechanism. Only two subunits 61.7 kDa UreB (subunit α) and 26.5 kDa UreA (subunit β) are encoded by ureB and ureA genes, respectively (Hamad, 2018). Three structural protein subunits; one large 61 kDa UreC (subunit α), 12.2 kDa UreB (subunit β) and 11 kDa UreA (subunit γ), are present in the case of *P. mirabilis* and encoded by ureC, ureB and urea structural genes respectively (Broll *et al*., 2021). The representation of different bacterial urease gene clusters and their protein products are presented in Fig. 2.

Helicobacter sp. urease have two structural genes and ureA genes have corresponding sequence with that of the hypothetical fusion of ureA and ureB genes of other bacteria and larger subunit ureB is analogous to ureC which contains the active center of the enzyme with two metal ions (Veaudor *et al*., 2019). In most of the ureases, the larger subunit is UreC except *H. pylori* has UreB larger subunit. The enzyme forms a complex structure having 12 subunits. Polypeptides α and β form trimer, and the N-terminal domain of β subunit are necessary for aggregation. Such four trimers form a tetrahedral structure and this complex structure facilitates the enzyme activity even in acidic condition while other ureases undergo non-reversible inactivation (Maroney and Ciurli, 2021). Fig. 3 shows the structural domain organization of urease: (1) *H. pylori* (2) *P. mirabilis* (3) Trimeric organization of urease.

Mainly ureases are nickel-containing enzymes, but iron-containing enzymes were also reported in *Helicobacter mustelae* (Proshlyakov *et al*., 2021). Inactive apoenzyme is found in all bacteria cells and some accessary proteins are required for the activation of enzyme urease. These accessory proteins are mainly UreD, UreE, UreF, UreG and UreH facilitates the transport of nickel ions into the cell and incorporation into the active center of the apoenzyme (Maroney and Ciurli, 2021). The presence of urea is necessary for activating *P. mirabilis* urease as they have a regulatory ureR gene that produces urea inducible regulator, which mainly controls the expression of other genes (Duran Ramirez *et al*., 2022). A "flap" unit has a structure helix-turn-helix motif mainly located in the α subunit and present in two forms, one is an active open position in which urea may enter at the active site where the reaction is being performed and another one is a closed position in which flap covers the active center and blocks it (Loharch and Berlicki, 2022).

SUBSTRATES OF UREASE

Urease enzyme was thought to be very specific to the substrate urea until 1960s but since then, several more substrate has also been reported, including N-methylurea, N-benzoylphosphoric triamide, N-(3-methyl -2-butenyl) phosphoric triamide, N-hydroxyurea, phenyl phosphorodiamidate, semicarbazide, acetamide, phosphoric triamide, diamidophosphate, phosphoramidate, formamide and other esters and amides of phosphoric acid, thioacetamide and thiourea but their hydrolysis rate is much lower than that of urea (Qin and Cabral, 2002).

ACTIVATION MECHANISM AND COMPLEX OF UREASE

Inititation of activation complex mainly starts with the first protein UreD which binds with the apo-urease oligomer and acts as a scaffolds protein for the further binding of proteins. Then a GTSase-activating protein UreF $(UreABC–UreD)_3$ binds with the starting proteins and forms a complex $(UreABC–UreDF)_3$ and UreG binding finalise the formation of the activation complex. After GTP hydrolysis, a nickel-binding chaperone molecule, UreE, delivers the metal ion to the activation complex, resulting in a further enzymatic reaction (Palombo *et al.*, 2017).

Some modifications are also reported to this traditional model. New activation models suggested that nickel ion bound UreE enhances the GTP-uptake by binding with UreG in the presence of magnesium ion and nickel ion also translocated to the UreG and then an apo-urease

Table 1. Biochemical and kinetic parameters of ureases (Qin and Cabral, 2002, Krajewska, 2009, Kappaun *et al.*, 2018).

Sources	Properties						
	Origin In organisms	**Molecular weight (M_w) (kDa)**	**Optimal temp (°C)**	**Optimal pH**	**Isoelectric point (pI)**	**Km value (mM)**	**Specific activity (µmol urea/ min mg protein)**
Bacterial urease	Free, cytoplasmic, extracellular	200 - 1060 kDa	35-70	5.25 – 9.0	4.3-6.0	0.2-32 mM	30.6- 180,000
Plant urease	Mainly cytoplasmic	175 - 540 kDa	35-65	4.5 – 8.2	5.0-5.1	0.12-3.6 mM	14.5-3500
Fungal urease	Cytoplasmicor Extracellular	180-540 kDa	35-70	8.0-8.5	5.5	1.03-4.1 mM	670-1750

complex formed Ni^{2+}-$(UreDFG)_2$. GTP hydrolysis by UreG, specifically catalysed by $KHCO_3/NH_4CO_3$ results in the activation of the urease enzyme (Menegassi *et al*., 2018). The pathway showing the activation mechanism of urease is shown in Fig. 4.

REACTION MECHANISM OF UREASE

By describing the mechanism of enzyme inhibition, the debated urease mechanism of action was explained (Krajewska, 2016, Mazzei *et al.*, 2017). Urea binds with nickel ions in urease active site via carbonyl oxygen by taking the place of water molecules W1-W3 and making the urea more electrophilic in nature and favours the necleophilic attack. Urea forms a bidentate bond in the reaction of nickel ions with one of its amino nitrogen atoms. This bond enables the nucleophilic attack on carbonyl carbon which results in the release of carbamate and ammonia and formation of tetrahedral intermediate. Benini *et al.* (1999) controversies the preceding statement and proposed that nucleophilic reaction is accomplished through the donation of protons by bridging hydroxide to NH_3. While Karplus *et al.* (1997) reported that the histidine molecule from the active site acts as a proton donor and water molecule as a nucleophile mainly provided by mono-dentate binding to Ni (1) with Ni (2) to urea. In spite of these suppositions a simplified computer model was proposed by Estiu and Merz, 2007 stated that both elimination and hydrolysis reaction mainly performed competitively and ‘‘protein-assisted elimination” is preferred among them. The interaction between urease and PAEs (Phthalic acid esters) like dibutyl phthalate (DBP) and dimethyl phthalate (DMP), which have potential biological toxicity, formed stable complexes increasing its rigidity and stability (Mazzei *et al*., 2020). The non-competitive activation mechanism increased the urease activity (Wenjing *et al.*, 2021). Fig. 5 shows the diagrammatic scheme of the reaction mechanism for urea hydrolysis by urease.

UREASE INHIBITORS

Enzyme inhibitors developed based on the molecular structure of the substrate is a method used in the design of rational drugs. Urease is sensitive to heavy metal ions even in trace amounts. These metal ions reacted to the sulfhydryl group in the active site of the enzyme just like the formation of metal sulfides resulting in its inhibition (Liu *et al*., 2018).

Insoluble sulfides formed after the reaction with metal ions resulted in the formation of strongest inhibitors, as Ag+ and Hg2+ can completely inhibit the enzyme activity even at 10^{-6} M concentration (Habala *et al*., 2018). Heavy metal ions like Fe^{3+} Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, Cu^{2+} and Hg^{2+} showed enzymatic inhibition in a non-competitive manner (Schafer *et al*., 2018). Some urea analogous substrates like boric acids, boronic acids, thiol reactive reagents, fluoride, alkylated ureas, thioureas, hydroxamic acids, phosphoroamides, hydroxyurea and thiols also inhibited the enzyme activity. Enzyme substrates of urea derivatives like hydroxyureas, thioureas and selenoureas and methylurea are also enzyme inhibitors. The inhibitory activity is also reported by aryl-alkyl hydroxamic acids, n-aliphatic, m- and p-substituted benzo (Rashid *et al*., 2020). Phosphoramidates (Di- and triamides of phosphoric acid) and hydroxyurea group demonstrated significant activity against urease enzymes (Hameed *et al*., 2019). Both these compounds rapidly degrade enzymatic activity and allow slow recovery of the enzyme after their hydrolysis due to their high degree of resemblance with tetrahedral transition state of the urea hydrolysis reaction (Santoro *et al*., 2020). Urinary and gastrointestinal infections of human can potentially be controlled and efficiency of urea fertilisers can be enhanced by urease inhibitors (Sarfraz *et al*., 2019).

The high oxidising potential of quinones makes their reactivity to sulfhydryl groups and high affinity with the cysteine residue of enzyme, so they also work as inhibitors of urease enzyme (Kappaun *et al*., 2018), and this mechanism is analogous to the inhibitory action of the α

-,β-unsaturated ketones, similar to the structure of quinones (Silakari and Piplani, 2020). polyhalogenated benzo- and naphthoquinones have also been reported to show inhibitory effect on urease (Silakari and Piplani, 2020). Benzoquinones substituted with chlorine (IC50 = 1 µM for p-chloranil) and fluorine (IC50 = 1.5 µM for p-fluoranil) showed strong potential against *H. pylori* urease, while naphthoquinone showed a strong inhibitory effect against *P. mirabilis* urease and O-napthoquinones against jackbean urease (Svane *et al*., 2020, Ghobadi *et al*., 2021). N-(n-butyl) thiophosphoric triamide (NBPT) and phenyl phosphorodiamidate (PPD/PPDA) have been shown to inhibit enzymatic activity (Huey et al., 2019), and barbituric analogues, phosphoramidated, and five to six-membered heterocycles have also been shown to inhibit enzymatic activity (Rego et al., 2018). Thio-barbituric acid (TBA), nicotinamide (NCA), barbituric acid (BTA), isoniazid (INZ) were also reported to suppress the enzymatic activity (Shah *et al.*, 2020). Extremely high acidic pH can denature the subunits of urease by decreasing its stabilising interactions, which is caused by an increase in mobility of the flaps (mobile regions) that covered the active sites of urease (Barazorda-Ccahuana *et al.*, 2020). Allicin compound extracted from freshly crushed garlic cloves is also reported to inhibit hydrolysis reaction of urease (Huey *et al.*, 2019). Hydroxamic acid and dihydropyrimidine derivatives perform mixed inhibition with both competitive and non-competitive mechanisms against urease enzyme (Mamidala *et al.*, 2021). Benzenesulfonohydrazides can be used as a potent urease inhibitor with competitive inhibition (Ahmed *et al.*, 2020). Dithiobisacetamides were also determined to function as urease inhibitors with mixed inhibition and nearly no cytotoxicity (Liu *et al.*, 2021). *Eucalyptus camaldulensis* , as well as *Vachellia nilotica* extracts showed significant inhibition potential against urease and can be used as a coating on urea prills to minimize hydrolysis of urea and its prolonged presence in soil (Rana *et al.*, 2021).

UREASE IMMOBILIZATION

Immobilized enzyme is the dormant form of the enzyme, attached to insoluble and inert material mainly responsible for the resistance to pH or temperature change and enzyme reuse (Zaushitsyna *et al.*, 2013). Thermal and operational stability of immobilised enzyme is greater than the soluble form of the enzyme (Wu *et al.*, 2013). Immobilization of an enzyme can be done by various ways including alginate matrix or beads, in a porous material, adsorption on glass, Entrapment in gel beads, using non-covalent or covalent protein tags, Cross-linkage and Covalent bonding (Cassimjee *et al.*, 2014, Zucca and sanjust, 2014). Urease was also immobilised using a composite film synthesised from electro-inactive polypyrrole with various compounds like polyaniline membrane, composite hydrogel membrane, alginate matrix coated by poly (methylene coguanidine), chitosan membrane, gelatin

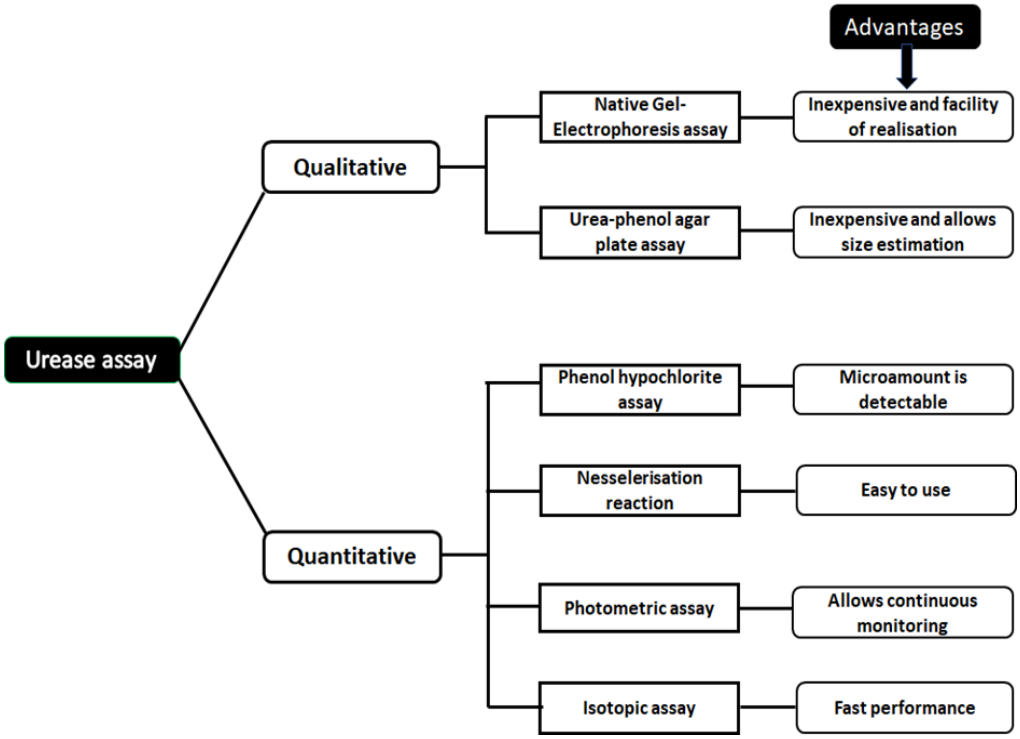

Fig. 1. *Different assays for detection of urease (Braissant et al., 2020, Toth et al., 2018, Dong et al., 2017, Hashihama et al., 2015, Nam et al., 2016, Dilrukshi and Kawasaki, 2016, Chang et al., 2017)*

Table 2. Some medical applications of urease

Sr. No.	Urease source	Medical application	References
1	*Helicobacter pylori* urease	Important drug target as it develops pathogenesis of many bacteria including *H. pylori*. Urease inhibitors alongwith antibiotic increase the efficiency of the drugs against bacteria.	Hameed *et al.*, 2019
		Activates non gastric cells like platelets, neutrophiles, endothelial cells to release cytokines for inflammatory reaction.	Kappaun *et al.*, 2018
2	Bacterial and fungal lungs pathogen	Rapid *in vivo* urease detection from lungs helpful in the diagnosis of lungs infection, ventilator associated pneumonia, tuberculosis and acute axacerbations of chronic bronchitis by inhaled ^{13}C breath test	Bishai and timmins, 2019
3	Human microbiota urease	Urea degradation in interstitial medium near vicinity of cancerous cells is cytotoxic and produce alkaline effect and increased pH enhanced the efficacy of anti-cancerous drugs like Vinblastin, doxorubicin and chemotherapeutic drugs	Rajendran *et al.*, 2018
		Urease and carbonic anhydrase enzyme promotes the mineralization hydrogels with calcium carbonate and magnesium carbonate and induce osteoblasts growth and bone regeneration	Douglas *et a*l., 2017
4	GI tract urease	High ammonia concentration due to urease activity damages the GI mucosa and results in the impairment of nutrient absorption, decreased growth, futile energy and protein spillage in ruminants	Patra and Ashenbach, 2018
5	Gut and oral microbes urease	Alkali (ammonia) production inhibits the dental activities and plaque formation	Svane *et al*., 2020
6	Virulence factor for *Mycobacterium tuberculosis*	Through surviving in host	Dakal *et al*., 2021
7	Virulence factor for the pathogenesis of *H.pylori*	By surviving and persisting in host, enhancement of inflammatory reaction, aggregation of blood platelets, tight junctions damaging and exhibiting the cytotoxic effect.	Nabavi-Rad *et al*., 2022
8	Microbial urease	Immobilized urease removes excess urea in dialysis system and portable artificial kidney with a zirconium oxide/zirconium phosphate ion exchange system	Zhu *et al*., 2020
9	Virulence factor for bacteria *Proteus mirabillis, Morganella morganni, Ureaplasma urealyticum*	Through precipitation of polyvalent ions in all and through damage of glycosaminoglycan layer exclusively in *P.mirabillis.*	Behzadi *et al*., 2020
10	*Cucumis melo* plant urease	Shows diuretic effect in medicines mostly used to lowers the blood pressure and increase the excretion of urine and sodium ions in urine	Hussain *et al*., 2021
11	Urease from jackbean, soybean and *Bacillus pasteurii*	Induces platelets aggregation through activating their enzymes, lipo-oxygenase derived eicosanoids and calcium channels	Broll *et al*., 2021
12	Microbial urease	Urease can acts as an antigen and activates strong immunological response. So urease can be used in vaccines for infection against some pathogenic bacteria like *H. pylori.*	Qiao *et al*., 2021
13	Microbial urease	Urease treatment, stable isotope dilution and GC-MS helps in significant diagnosis of homocystinuria I, II, and III by the detection of methionine, homocysteine, cysteine, creatinine, uracil, methylmalonate and orotate in human urine at prior stage increases the treatment efficacy	Phipps *et al*., 2019
14	Virulence factor for *Yersinia enterocolitica*	Through the activation of inflammatory mechanism	Lin *et al*., 2022
15	Urease based ELISA	By the utilization of novel monoclonal antibodies, Urease based ELISA is used for the confirmed detection for bacteria *Neisseria gonorrhoeae*	Garcia Gonzalez *et al*., 2022

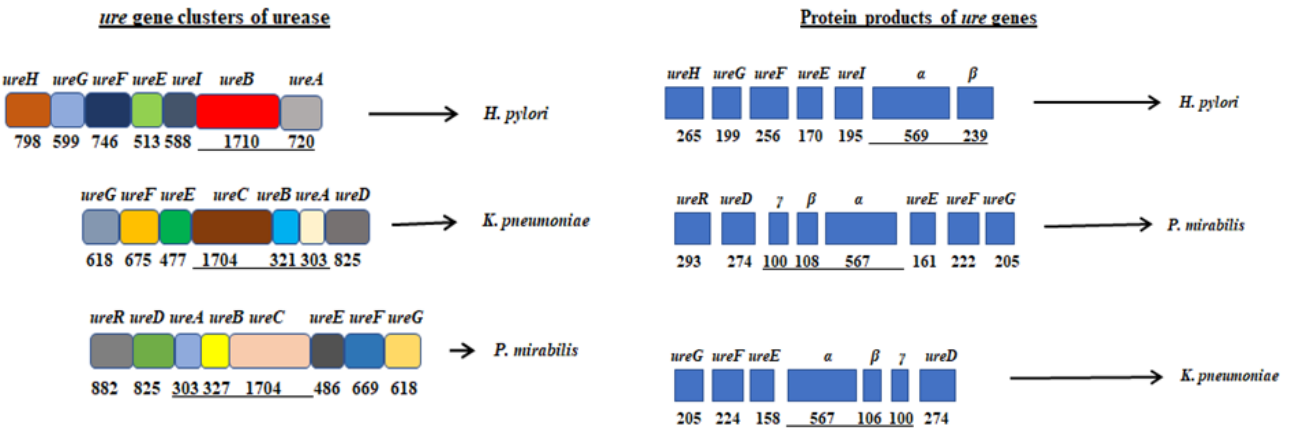

Fig. 2. *ure gene cluster and protein products of urease (Konieczna et al., 2012)*

membrane, chemically grafted nylon membrane, poly ion complex, thiophene-capped poly(methyl methacrylate)/ pyrrole matrix, poly(N-vinylcarbazole) membrane, polyvinyl alcohol activated membrane (Kutlu *et al*., 2020), PVC-NH2 membrane (Verma *et al*., 2020), non-porous HEMA incorporated poly(EGDMA) microbeads (Hussain *et al*., 2021).

Clinically applied biosensor synthesised in various polymers like lactobionate acetate and hydroxy groups for optimization of enzyme membranes. Acetate group cationic polymers possess high operational stability with any type of cross-linking (Kamel and Khattab, 2020). Gel entrapment techniques with various mixtures like polyacrylonitrile membrane, Zn–Al layered double hydroxide matrixes (Vijayamma *et al*., 2020), sodium alginate (San, 2019), crystalline colloidal array matrix (Noh and Park, 2018), thiol and alkyl thiol functionalized MNPs (Jangi *et al*., 2020) and acrylonitrile copolymer membranes (Hussain *et al*., 2021).

Immobilization can be done by using cyanuric chloride as well as phthaloyl chloride. This immobilized urease has superior retention ability on heating up to 100^0C. Urease immobilised on Teflon or lipid coated silica has maximum activity loss even after 1 hour of boiling in an aqueous solution (Shallsuku and Kariuki, 2021). Green synthesis of gold nanoparticles capped with cysteine (Cys-AuNPs) using leaf extract of *Salvadora persica* and using glutaraldehyde as cross-linker, urease can be immobilized on these particles. This immobilized urease seems to have 10 cycles of reusability and 35 days of storability with 50% residual activity (Singh *et al*., 2021).

Co-precipitation and coating phosphonomethyl iminodiacetic acid of magnetite nanoparticles through a carbodiimide reaction are also helpful in immobilization of urease. Enzyme in immobilized form has commendable catalytic activity and almost six times reusable and ultimately reduce the cost of the enzyme (Sahoo *et al*., 2011). Cross linking of alginate with enzyme lyophilisates (CLELs), (Akkas *et al*., 2020), diethylaminoethyl (DEAE)-cellulose strips (Kayastha, 2019), cation exchange resin (Al-Shams *et al*., 2020) and Ba++ ions (Saxena *et al*., 2017) efficiently immobilized the enzyme and used in the formation of biosensor useful in medical diagnostics.

APPLICATIONS OF UREASE

Enhancement in fertility of soil

Urease is abundantly present in soil, plants and bacteria. As nitrogen is a limiting factor for the growth of plants and the main physiological contribution of urease to plants is the conversion of urea into ammonia and carbon dioxide and increase the availability of nitrogen to the plants (Kappaun *et al*., 2018). The cell free urease also affects the availability of nutrients to the plants by increasing the alkalinity of the soil and inducing calcium carbonate (Krajewska, 2018).The high level of soil urease may decrease urea fertilization efficiency and in turn increases the ammonia loss to atmosphere and decrease ammonia induces phytotoxicity (Byrne *et al*., 2020). The ureolytic action of ureases increases the nitrogen availability by minimizing the crop damage caused by urea fertilization (Kumari *et al*., 2016) and recycling nitrogen bound to urea during the develop-

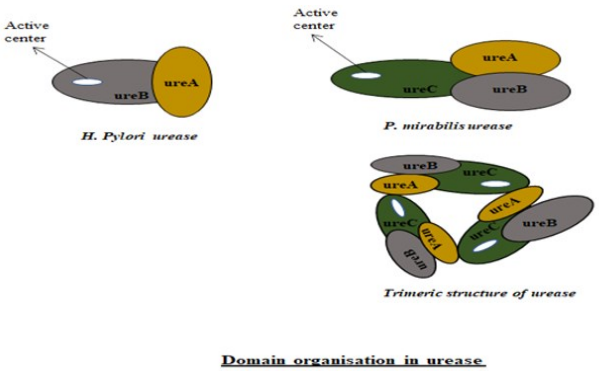

Fig. 3. *Domain organization in urease: 1. H. pylori urease 2. P. mirabilis urease 3. Trimeric structure of urease (Konieczna et al., 2012)*

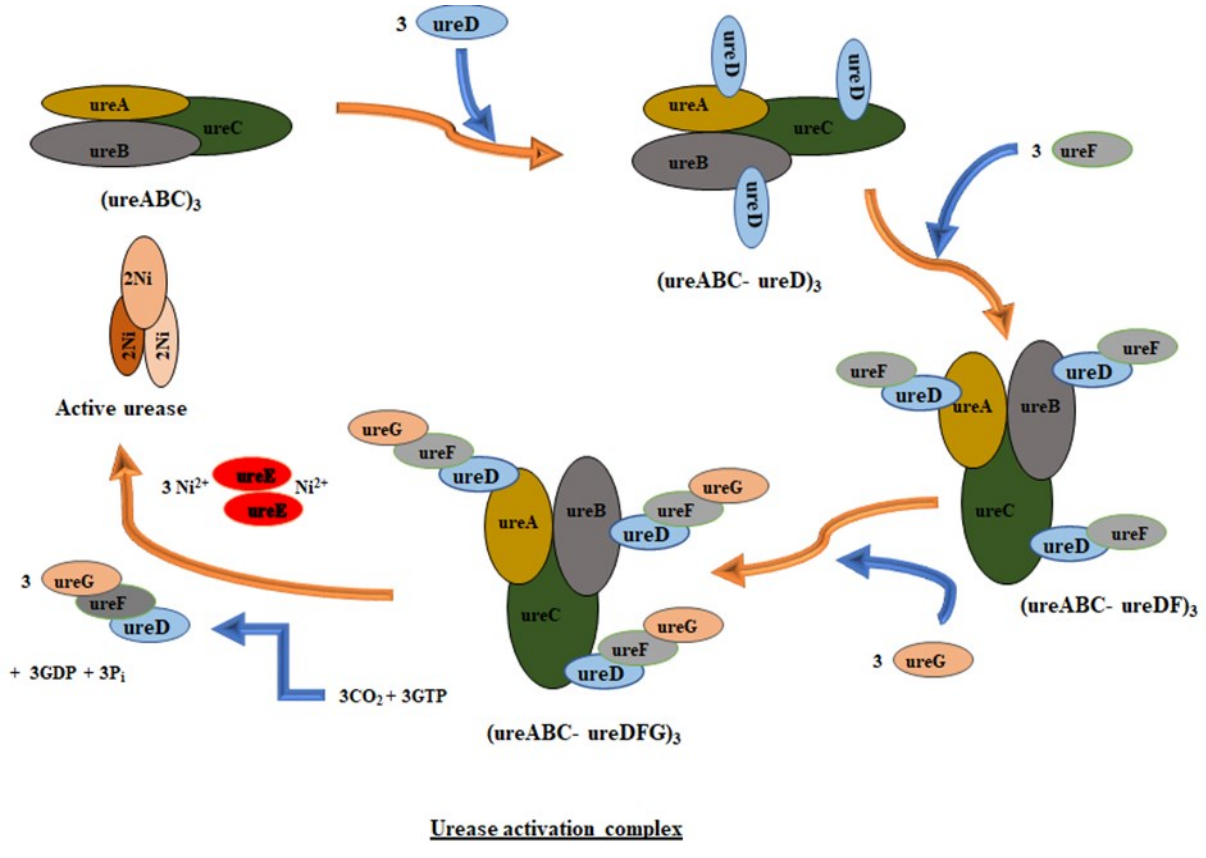

Fig. 4 *Activation complex of urease (Quiroz-Valenzuela et al., 2008)*

ment of seedlings (Hussain *et al*., 2021).

Role in cell to cell/organism communication

Arginase from lichens *Evernia prunastri* and *Xanthoria parietina* with lectin properties showed binding with glycosylated urease (acts as ligand) present in cell wall of homologus algae. This binding may reveal a model of recognising compatible algae, through which algal cells would form lichen with lectin secreting fungus. This property can be used for cell to cell/organism to organism communication (Tran-Trung *et al*., 2020).

Contribution of urease to plant defense against predators and pathogens

One or more protein domain, distinctly present in active site of Jackbean and soybean urease showed entomotoxic activity. JBTX and CNTX toxins extracted from *Canavalia ensiformis* inhibited many insect species (Sa *et al*., 2020, Kappaun *et al*., 2018). Knock down of PLA2 (PhospholipasesA), cathepsin, Dexamethasone, indomethacin and calcium chelation inhibitors mainly inhibit these insects (Deferrari *et al.*, 2014a, Deferrari *et al.*, 2014b). Ureases also have pro-inflammatory, endocytosis-inducing and neurotoxic activities that do not require ureolysis. Ureases are particularly relevant in plants for exerting insecticidal and fungitoxic effects (Kappaun *et al.,* 2018).

Medical application of ureases

Urease-dependent antibiotic-resistant bacteria were listed in the priority pathogen list of WHO for research and development of new antibiotics, as some of them are supposed to cause bacterial infection of the respiratory tract (Mazzei *et al.*, 2021). Moreover, half of the patients who died of COVID-19 in Wuhan (China) were co-infected with these type of bacteria in the lungs (Zhou *et al.*, 2020). Urease enzyme-powered polymer nanomotors can be used for drug delivery and treatment of several bladder related diseases (overactive bladder, bladder cancer, bladder cystisis etc.). After injecting, these urease nanomotors can penetrate deep inside the mucosal layer of the bladder with a long term period to remain in the bladder even after repeated urination. They become active, move around in the bladder and convert urea to CO_2 and NH_3. This can become a rapid and efficient approach for intravesicular therapeutic delivery (Choi *et al*., 2020). Inhibitory mechanisms involving the structural similarity of the substrate through modification or derivatization can also help in rational drug design. It can be performed by two possible competitive ways either by mimicking monodentate urea binding or binding as a tetrahedral intermediate displaying good steric and chemical complementarily with the ligand binding site (Hamad *et al*., 2020). Further applications, in brief, are given in Table 2.

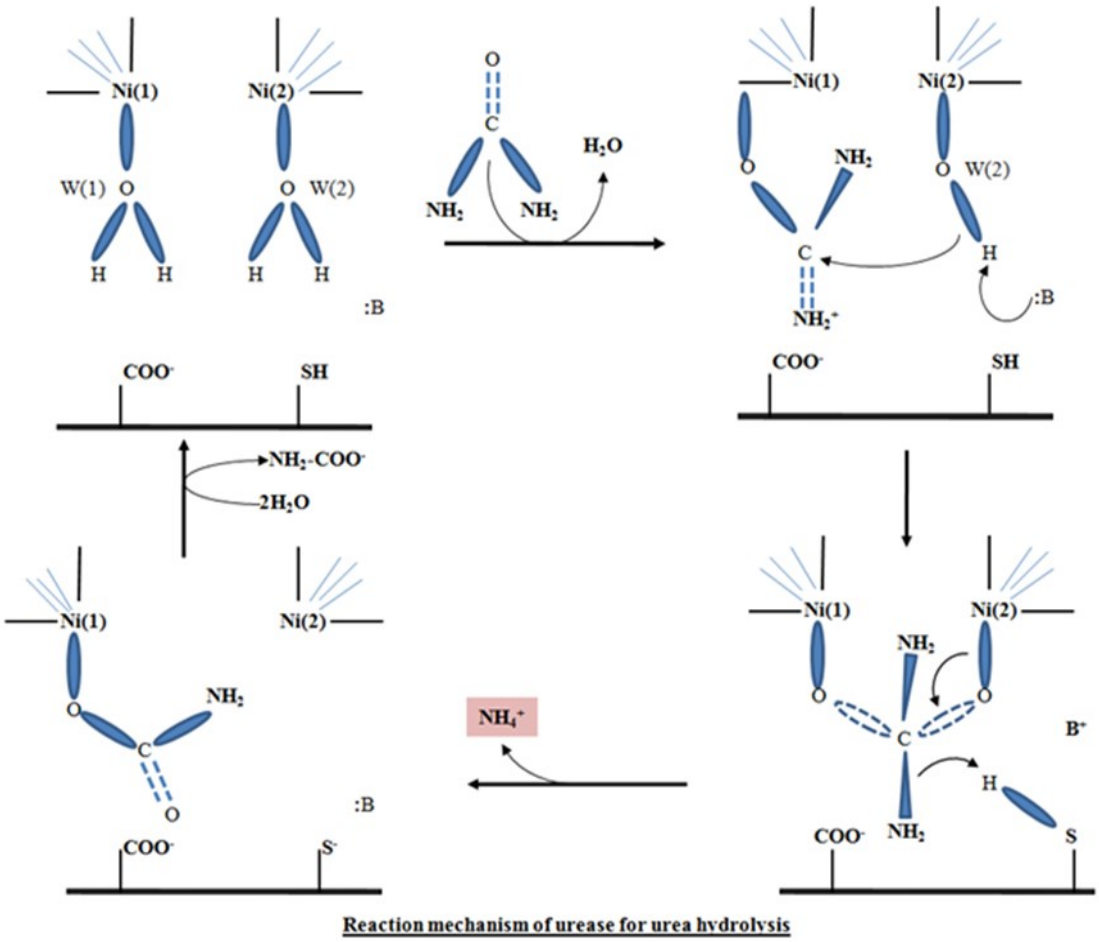

Fig. 5. *Reaction mechanism for the activity of* urease (Mazzei et al., 2020)

Analysis of urea concentration

Biosensors based upon urea nanoparticles are generally prepared by glutaraldehyde crosslinking, and desolvation and mainly functionalized by cysteamine dihydrochloride and these biosensors are mainly used to determine urea concentration in wastewater, wines, foods, blood serum and urine (Saeedfar *et al.*, 2013). Also, the gold nanoparticles synthesized from urease function as a conductometric biosensor for analysis of urea concentration. Many transductive technologies like amperometric, potentiometric (pH electrodes, ammonia or carbon dioxide gas-selective electrodes, field-effect transistors ammonium ion-sensing electrodes), optical, conductimetric, acoustic, spectrophotometric (Saeedfar *et al.*, 2013), calorimetric (Fatoni *et al.*, 2019) and ion-pair liquid chromatographic methods (Wang *et al.*, 2016) were used for analysis of urea. Highly luminescent ZnS quantum dots bio conjugated with immobilized urease via amide bond act as a pH fluorescent label for urea determination. The change in pH due to alkaline conditions after enzymatic hydrolysis of urea resulted in deprotonation of ZnS QDs and an increase in the fluorescence intensity. This increased fluorescence density helps in bioassay of urea concentration (Safitri *et al.*, 2017).

Urease containing hydrogel can be used in designing and fabricating a secure information protection system. Fluorescent hydrogels can be used to input information based on protonation of 4-(N,N-dimethylaminoethylene) amino-N-allyl-1,8-naphthalimide (DEAN-H^+) in conjunction to dopping with enzyme (urease) with metallic ions like $Zinc^{2+}$ coordinated to DEAN. When this system is exposed to urea, ammonia is produced resulting in decreased fluorescence in a hydrogel that revealed the information. In few minutes, the information displayed gets deleted by itself (Le *et al.*, 2021).

Analysis of heavy metals

Urease is mainly used to check the level of heavy metal ions in waste water, soil extract, drinking water ground water and surface water. The function group at active site of urease main interacts with heavy metal ions. Mass transfer limitations are the main factors that create variations in urease activity and can be applied in biosensor development (Fopse *et al.*, 2019). Heavy metals such as Pb and Cd that are toxic to organisms can be extracellularly adsorbed/immobilized and increasing pH can also protect urease-producing bacte-

ria. This urease-producing property can be applied for vegetable safety and in situ bioremediation of heavy metal from polluted land (Wang *et al.*, 2020).

Environment pollution control

Urease producing bacteria enhance the mineral precipitation process which is helpful in the removal and inertisation of heavy metal ions in waste and soil (Kang and So, 2016, Arias *et al.*, 2017). A mixed culture of urease-producing bacteria (*S. pasteurii* and *B. cereus*) caused pulverization of coal using a calcium and urea source which might have resulted in coal dust suppression (Zhu *et al*., 2020). Bio-leeching induced by bacterial urease, their organic products and their potential for carbonates enhanced leeching of Ca, Mg, Mn and V, which would benefit the recycling of metals. Bio-processed sand mixtures and coal fly ash can be utilized for construction materials (Zhang *et al*., 2021).

Role in geological formation and geotechnical engineering

Soil calcium carbonate precipitation resulting from ureolysis done by urease improves sand/soil strength and controls soil permeability (Park *et al.*, 2014). The negatively charged urease producing bacterial cells adsorb $Ca^{2+,}$ which can be used as a nucleation site for accelerating the formation of $CaCO_3$ (Yi *et al.*, 2021).

It is also involved in the construction of subsurface barriers that prevent the mixing of salt water with fresh water bodies and dust treatment (Phillips *et al.*, 2013). Calcium carbonate mineralization after ureolysis is effectively used to clean the water from heavy metals and radio-nuclides through solid phase capture (Anbu *et al.*, 2016, Kumari *et al.*, 2016), to remove calcium from wastewater of industries, citric acid production, severe scaling in reactors and pipelines, citric acid productions and bone processing, and paper recycling (Krajewska, 2018). This bio-processed $CaCO_3$ is also used in encapsulation of polychlorinated biphenyls and removal from contaminated oil (Singh *et al*., 2021). Fluorescent calcite synthesized from bacterial urease is used as a filler in plastics and rubbers, fluorescent particles in stationery ink (Gwenzi, 2019) and fluorescent marker in biochemical applications (Mostafa *et al.,* 2021). *Lysinibacillus fusiformis* produced urease that enhanced the concrete strength of crushed cubes (Hussaini, 2021).

Also, soybean urease makes the pore size distribution more uniform, which increases the $CaCO_3$ precipitation resulting in a hike in the desorption rate of the water retention capacity of sand (Chen *et al*., 2021).

Beverages and wine industry

Urea is considered as main precursor for ethyl carbamate (EC) production in wines and beverages which is a harmful carcinogen. Reduction of urea from beverages could successfully reduce the EC amount (Yang *et al*., 2021). Several physical methods like refining rice in sake formation, supplementing diammonium phosphate in the beginning step of fermentation in wine formation, lowering the temperature and speeding up the reflux rate distillation in sugarcane spirits and charcoal filtration in diluted spirits lower the ethyl carbamate level very significantly. Some chemical methods, such as copper catalysis in stone-fruit spirits formation, and adding potassium metabisulfite in 'ume' liquors formation, also yielded the same results (Zhao *et al.*, 2013). This urea elimination process mainly includes shaking and incubation through refining and complete inactivation is mainly done by pasteurization process. Acid Ureases from *Lactobacillus fermentum*, *Arthrobacter mobilis*, *Enterobacter* sp., *Lactobacillus reuteri* etc. had been reported and characterized for urea degradation in beverages (Liu *et al*., 2019). *Escherichia coli* mutated with urease had shown significant degradation of EC and urea in rice wine. This strategy can be used further to reduce microbially metabolized ammonia hazards in fermented foods (Jia and Fang, 2020).

Conclusion

Urease is gaining remarkable attention due to its diverse range of application for mankind. It is already an attractive drug target for designing anti-infective agents for pathogens like *Proteus mirabilis*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Campylobacter pyloridis, Helicobacter pylori*, *Klebsiella* species, *Salmonella* sp. etc. Recent research mainly comprises of exploring novel urease inhibitors and immobilization techniques. The recently developed synthetic inhibitors like Benzenesulfonohydrazides and dithiobisacetamides derivatives and the natural plant extracts of *E. camaldulensis* and *V. nilotica* are having significant inhibition potential with almost no cytotoxicity. These can be used as urea coating to minimize its hydrolysis in soil. The studies concluded that findings for novel natural products to inhibit the urease enzyme with no side effects can be achieved in future. The use of urease immobilized on green synthesized nanoparticles like Cysteine coated AuNPs and phosphonomethyl iminodiacetic acid coating of magnetite nanoparticles has been increased due to its high residual activity and reusability with cost effectiveness. Its property of entomotoxicity can be utilized for insecticidal and fungicidal effects to protect crops in upcoming times. The development of urease nanomotors has opened a new path in the field of drug delivery and treatment of bladder-related infection and disease. Urease based biosensors are getting keen interest to analyse the presence of heavy metals and urea. The urease hydrogel is a recent development that may prove beneficial for design-

ing and fabricating secure information protection systems in future. Its role in the bioremediation of toxic heavy metals can further make it an interesting approach to reducing environmental pollution. Its role as urea and ethyl carbamate degrader in fermented products makes it a good tool to reduce hazards in beverages and fermented foods. Studies related to its controversial structure prediction and reaction mechanism would also be a research area in future. Its hidden role in different fields and structure prediction of uncharacterized urease from different sources can also be scope for researchers in future.

Conflict of interest

The authors declare that they have no conflict of interest.

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