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RESEARCH PAPER

Optimization of urease production by *Bacillus halodurans* PO15: a mangrove bacterium from Poovar mangroves, India

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Abstract

Mangrove ecosystems are one of the most versatile habitats for microorganisms with a high potential for producing a variety of extracellular hydrolytic enzymes. In this study, bacteria with urease activity, enzymes that catalyze the hydrolysis of urea into carbon dioxide and ammonia, were isolated from mangrove sediments of Poovar (Trivandrum, India). *Bacillus halodurans*, strain PO15, isolated in this study with high urease (UA) activity (28 U/ml) was subjected to optimization using a Box-Behnken experimental design. Incubation variables included incubation period, pH, inoculation percentage and temperature. Significant factors identified based on the model were incubation period, pH, incubation temperature, and inoculum percentage; variations in these produced a tenfold increase in UA activity (295.80 U/ml). The specific activity of the purified UA enzyme was 62.34 U/mg and was found to be thermostable (active up to 60 °C). UA of *B. halodurans* PO15 has potential for microbial-induced biomineralization with a reduction of free Ca^{2+} to about 82.8% ± 0.17%. The microbial-induced calcium precipitation (MICP) using the UA enzyme will potentially be beneficial in the process of biomineralization as well as for a variety of industrial applications.

Keywords Urease · Biomineralization · Optimization · Mangrove · Enzymes

Introduction

Microorganisms play a major role in ecosystem engineering through several biogeochemical process (Graham et al. 2016). These processes are mostly driven by microbial enzymes. Microbial enzymes have been well studied for their industrial applications, and environment management. Urease, secreted by bacteria, is one such enzyme that has numerous applications. Urease is extensively used as a diagnostic tool in the detection of urea in blood (Smith et al. 1993), in alcoholic beverages to remove urea, biosensors for detection of heavy metal ions, and biocalcification (Sarda et al. 2009). Microbial ureases can induce calcite precipitation through reaction of urea and free calcium ions, a function that has applications in civil and geotechnical engineering for enhancing the strength and stiffness properties of soil through the process of biomineralization (Anitha et al. 2018; Bibi et al. 2018; Cheng and Cord-Ruwisch 2013; Ivanov and Chu 2008).

Urease (EC 3.5.1.15) is a nickel-containing enzyme which catalyzes the formation of carbon dioxide and ammonia from urea (Cheng and Cord-Ruwisch 2013) resulting in an increase in pH in the surrounding media (Mora and Arioli 2014). Urease-producing microorganisms are relevant to human microbiota, which hydrolyses urea (Chen and Burne 2003; Morou-Bermudez and Burne 2000; Wegmann et al. 2013; Yatsunenko et al. 2012). The production of urease enzyme by ureolytic bacteria in soil may be influenced by a variety of factors. It is induced in the presence of urea and inhibited in the presence of ammonia and nitrogen compounds (Mobley et al. 1995). Several studies have reported the isolation of indigenous ureolytic bacteria, using enrichment cultures from soil, ground water and cement samples (Achal and Pan 2011;

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Burbank et al. 2012; Elmanama et al. 2013; Hammes et al. 2003; Rivadeneyra et al. 1993). Urease has been identified as a virulence factor for several microbial pathogens (Mora and Arioli 2014). Its role in microbial infections was well established through studies on *Helicobacter pylori* (Mora and Arioli 2014). The urease activity of microorganisms, such as *Proteus mirabilis* and *Staphylococcus saprophyticus,* plays a vital role in urinary tract infections through struvite–carbonate–apatite urinary stone formation. Based on the urease properties on formation of carbonate apatite stones, this enzyme has an application in environmental engineering. In addition to the above, urease enzyme also has many medical applications, including use as new drug targets.

Despite their application in the fields of biomedical and environmental engineering, the production conditions of the urease enzyme remain poorly understood. Previous studies have been limited to one-factor optimization (Bakhtiari et al. 2006; El-Bessoumy et al. 2009) and have not concluded suitable production parameters for enhancing urease activity. For industrial applications, it is important to determine the optimal conditions for enzyme production. Traditionally, factorial methods were used in kinetic studies on microbially induced carbon precipitation (MICP) but this method is costly and time-consuming. Methods such as response surface methodology (RSM) in the form of the Box-Behnken experimental design were used to determine the optimum levels of key conditions as determined by Plackett–Burman design (PBD) (Box and Behnken 1960; Plackett and Burnam 1946). This method could overcome the challenges of the conventional optimization techniques, which are laborious and result in unreliable and inaccurate results. These statistical techniques could help in designing experiments, building models, evaluating the interactive effects of variables, and determining optimum conditions (Shivam et al. 2009). RSM is widely used in bioprocessing technology for optimization of fermentation media (Desai 2008; Rishad et al. 2016; Sunitha et al. 1999). In a study by Khodadadi and Bilsel (2015), a central composite face-centered (CCF) design was used to fit a second-order model to evaluate microbial urease efficacy in the biocementation process. Optimum conditions of enzyme-specific rate and urea hydrolysis were found to be significant. RSM or statistical modelling has been employed in the optimization of several enzymes, due to their reliability (Ameri et al. 2019; Nathan et al. 2018; Raza et al. 2019; Vijayaraghavan and Vincent 2014). This approach resulted in greater enzyme production of the microbial strains and are essential for any industrial applications. In this paper, we focus on a urease-producing bacterium, *Bacillus halodurans* PO15, isolated from mangrove sediment. We used statistical models for optimizing urease production at different incubation periods, pH, inoculation percentage and incubation temperatures, to achieve maximum enzyme activity. The biomineralization ability of the urease was also evaluated. This is the first report of urease optimization through statistical models.

Results and discussion

Screening of urease-producing bacteria

Fifty-two bacterial cultures were isolated from Poovar mangrove ecosystem sediment samples. Of these, 21 isolates gave urease positive results on urease agar. These

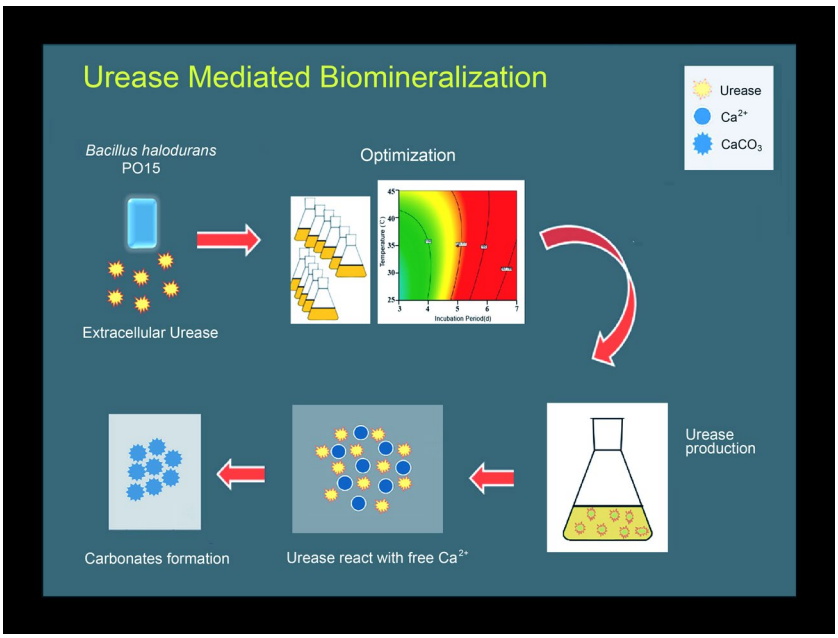

Fig. 1 Representation of urease production using *Bacillus halodurans* isolate PO15 based on Box-Behnken experimental design

bacterial strains were inoculated into urease broth and urease production was quantified, based on a spectrophotometric assay. The urease activity of these isolates ranged from 1.8 to 28 U/ml. The bacterial isolate with the highest activity was selected for the further experiments. The strains with high urease activity were identified as *B. halodurans* through 16S rRNA ribotyping. The urease-producing bacteria is ubiquitous in natural environments. However, other common urease-producing strains reported here, *Helicobacter pylori*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, etc., are pathogenic or opportunistic pathogens to humans (Stabnikov et al. 2013). Additionally, many other bacterial strains that are used in microbial-induced calcium precipitation (MICP) with urease production *Bacillus* sp. VS1 and *Bacillus* sp. were reported (El-Bessoumy et al. 2009; Stabnikov et al. 2013). VUK5 has been extensively used in MICP studies (Stabnikov et al. 2013). In biocementation studies, spore-forming strains of urease-producing bacteria, were found to be more compatible to environments with high salt concentrations (Bachmeier et al. 2002; Stabnikov et al. 2013). For this reason, the optimization experiment in the present study was conducted for the *B. halodurans,* isolate PO15 alone. The major concern for environmental applications is the selection of an avirulent bacterial that has no adverse effect on humans or animals. Though many high urease-producing bacteria have been reported, they are mostly associated with human pathogenesis and cannot be used for any in situ environmental applications. In the present study, the mangrove bacterium, *B. halodurans* strain PO15, has no reported virulence and so could be employed in the optimization analysis.

Statistical optimization for urease production

In the present study, the most promising urease-producing isolate (*B. halodurans,* isolate PO15) was selected, based on urease activity. Figure 1 presents a schematic representation of urease production in the *B. halodurans* isolate P015, based on Box-Behnken experimental design. Initial urease activity of *B. halodurans* isolate PO15 was about 28 U/ml. This value was quite higher than the activity reported for *Bacillus thuringiensis* N2, a marine bacterium (3.53 U/ml) (El-Bessoumy et al. 2009). To determine the effect of different factors on urease production, culture conditions were optimized using a Box-Behnken experimental design. A maximum urease activity of 295.80 U/ml, a tenfold increase from initial activity, was achieved using the design (Table 1). Significant factors identified,

Table 1 Optimization of urease production using *Bacillus halodurans* isolate PO15 based on Box-Behnken experimental design

Run	Actual Val	Predicted Val
1	234.05	242.13
2	258.91	239.63
3	202.08	208.55
4	225.25	205.97
5	234.05	242.13
6	209.50	177.22
7	231.50	250.97
8	234.05	242.13
9	234.05	242.13
10	234.05	242.13
11	234.05	242.13
12	234.05	242.13
13	234.05	242.13
14	234.05	242.13
15	241.41	211.13
16	234.05	242.13
17	234.05	242.13
18	248.50	267.97
19	300.66	268.38
20	234.05	242.13
21	267.25	284.72
22	234.05	242.13
23	234.05	242.13
24	298.58	279.30
25	220.75	238.22
26	248.58	266.05
27	176.83	196.30
28	233.83	253.30
29	204.75	211.22
30	262.91	243.63
31	253.41	223.13
32	316.66	284.38
33	234.05	242.13
34	289.25	295.72
35	268.75	238.47
36	234.05	242.13
37	326.08	295.80
38	234.05	242.13
39	234.05	242.13
40	234.05	242.13
41	162.08	179.55
42	270.83	238.55
43	246.58	253.05

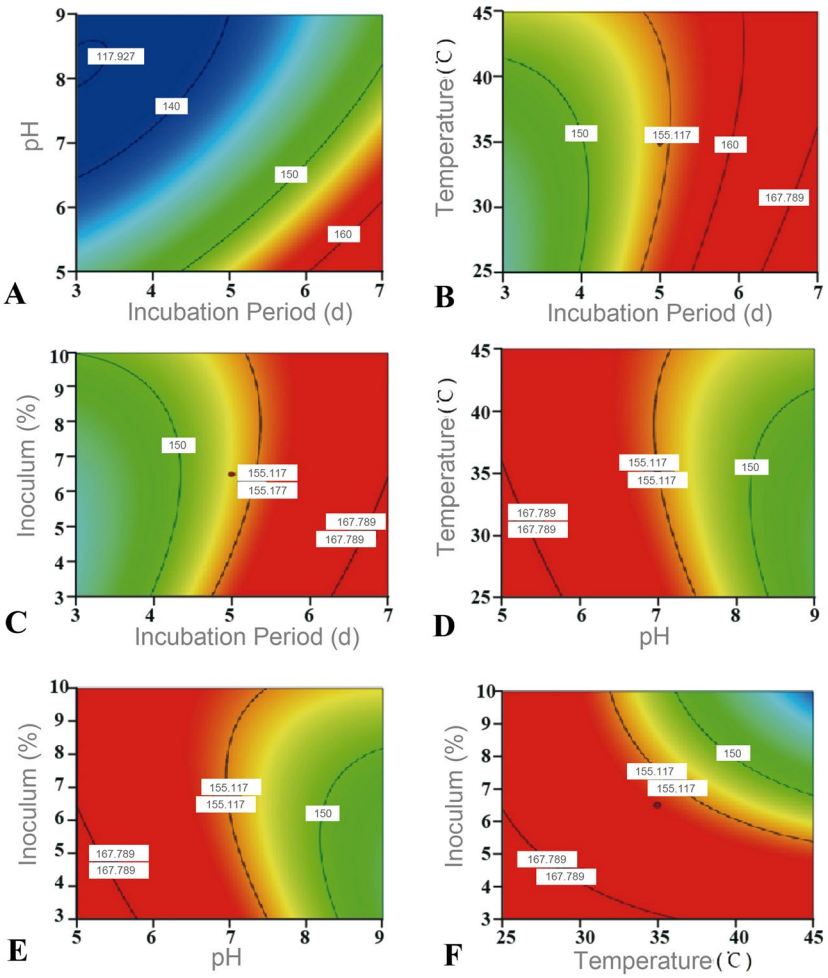

Fig. 2 Contour plot showing response of variable influencing urease production using *Bacillus halodurans* isolate PO15 based on Box-Behnken experimental design. The interaction between variables are shown: **a** incubation period vs. pH; **b** incubation period vs. temperature; **c** incubation period vs. inoculum (%); **d** pH vs. temperature; **e** pH vs. inoculum (%); **f** temperature vs. inoculum (%)

based on the model, were the incubation period, pH, incubation temperature, and inoculum percentage. Though aeration was reported as a major factor in enzyme production, for urease production, oxygen concentration has no role except in MICP (Bakhtiari et al. 2006). The design predicted from the experiment was found to be significant (R^2 value of 0.9961). Equation 1 represents the quadratic model regression equation describing the predicted model. Khodadadi and Bilsel (2015) reported that the conditions favouring the urease production of *S. pasteurii*, the amount of urea hydrolyzed, and the rate of hydrolysis all inhibited bacterial cell growth and the specific hydrolysis rate of urea and vice versa. However, in this study, a better urease production using *B. halodurans* PO15 was achieved. However, it is difficult to draw a comparison with other studies as most of them reported on the urea hydrolysis rate rather than urease production.

The fitted model is represented as Eq. 1

$$\begin{aligned}\text{UA activity (U/ml)} &= 355.315 + 80.2708 \times \text{Incubation Period} \\ &+ (-35.8631) \times \text{pH} + (-7.31012) \\ &\times \text{Temperature} + 7.14286 \times \text{Inoculum \%} \\ &+ 0.125 \times \text{Incubation period} \\ &\times \text{pH} + (-1.7125) \times \text{Incubation period} \\ &\times \text{Temperature} + (-4.75) \times \text{Incubation period} \\ &\times \text{Inoculum \%} + 1.25 \times \text{pH} \times \text{Temperature} \\ &+ (-2.64286) \times \text{pH} \times \text{Inoculum \%} \\ &+ 0.921429 \times \text{Temperature} \times \text{Inoculum \%}. \end{aligned} \quad (1)$$

The interaction between the various medium components and factors for achieving maximum urease production are shown in contour plots (Fig. 2). From the interaction between the variables, it was found that the pH of the production medium is a critical factor for urease production.

When the medium pH vs. incubation period was tested, maximum urease activity of ~ 140 U/ml was achieved on the 6th day of incubation. For temperature vs. incubation period, urease activity reached a maximum after the 5th day only. Inoculum percentage vs incubation period achieved a maximum activity of 167 U/ml after 5 days. This suggests that a minimum of 5 days of incubation with a pH 5–7 is ideal for urease production, irrespective of all tested inoculum percentages and incubation temperatures. In another study on urease optimization of *A. niger* PTCC 5011, using conventional one-factor method, a maximum activity of 2.44 U/ml was obtained (Bakhtiari et al. 2006). Significant factors identified, based on the model, were incubation period, pH, incubation temperature, and inoculum percentage. The production of the enzyme depended on process variables such as nutrients, pH, temperature, incubation period, inoculum level and inducer concentration (Sharma et al. 2009). Optimization of medium by the classical methods involved changing one independent variable (i.e., nutrient, pH, temperature) while keeping all other variables constant. However, one-factor optimization is extremely time-consuming, expensive for a large number of variables (Okyay and Rodrigues 2014) and often results in wrong conclusions. Use of statistical-based approaches, such as response surface methodology (RSM), could overcome the limitations of the single-factor optimization process. Also, the RSM-based approach requires fewer trials to calculate the different variables and their interactions, compared to other optimization methods (Managamuri et al. 2017; Peng et al. 2018).

Except for the orthogonal array design-based approach for urease production using *A. niger* (Bakhtiari et al. 2006) and response surface methodology (RSM) (Khan et al. 2019), there are no statistical optimization-based reports available for other bacteria. This is the first report on the statistical optimization of urease production of *B. halodurans*. From the ANOVA results, the model F value of 6.65 and the *P* values < 0.005 indicate that the model was significant (Supplementary Table S1). The statistical model was validated through detection of 295 U/ml urease activity with optimized factors. Recently, El-Bessoumy et al. (2009) reported extracellular urease production from *B. thuringiensis* N2, however, the enzyme activity was very low.

Purification and characterization of UA

A specific activity of 62.34 U/mg was observed for purified urease with 5.6-purification fold and a yield of 87%. The specific activity of the isolate PO15 was higher than those derived from *Aspergillus creatinolyticus* (32.74 U/mg), *Lactobacillus reuteri* (13.0 U/mg) (Kakimoto et al. 1989), *A. niger* (0.325 U/mg) (Smith et al. 1993), and *R. oryzae* (0.18 U/mg) (Geweely 2006). Based on the LB plot, a V_{max} of 333.33 mmol L^{-1} mg^{-1} min^{-1} with K_m values of 1.7 mmol/L was observed for UA. The LB plot showing the enzyme kinetics is shown in Fig. 3a. The UA enzyme was tested for its thermostability and was found to be stable up to 60 °C (Fig. 3b). In another study, the fungal urease of *Aspergillus* exhibited maximum production and urease activity at 35 °C and the least activity was at 50 °C (Khan et al. 2019). Others reported 35 °C and 40 °C as the optimum temperature for urease activity (Danial et al. 2015; Fathima and Jayalakshmi 2012). In this study, however, a maximum urease activity at 35 °C was observed, the enzyme had no significant decrease in urease activity even at 60 °C. This clearly validates the thermostable property of the urease enzyme.

Similarly, the optimal pH for maximum urease activity was found to be pH 7 (Fig. 3c). There was an increase in urease activity with an increase of pH from 3.0–9.0 (Khan et al. 2019). Some bacterial ureases exhibited high activity in alkaline conditions (pH of 9.0) (Phang et al. 2018), while some had maximum urease activity at pH 8 (Danial et al. 2015; Mirbod et al. 2002). Two fungal isolates of the genus *Aspergillus* had an optimum pH of 8.0 and 8.5 (Kappaun et al. 2018). In general, the fungal urease had their maximum activity in the basic medium, while bacterial urease tended to be more variable (Khan et al. 2019). The higher thermostability favours the application of UA in a variety of industrial and environmental engineering applications.

Urease-mediated calcium precipitation

To understand the rate at which CO_2 is trapped as carbonates, a calcium carbonate precipitation study was carried out. The relative reduction of free calcium in the media is shown in Fig. 3d. *B. halodurans* PO15 was able to achieve a reduction of (82.8 ± 0.17)% free Ca^{2+}. Maximum reduction was observed after 48 h incubation. For the bioremediation of CO_2, the microbial biomineralization ability is of great importance (Silva-Castro et al. 2015). Application of urease derived from *Sporosarcina pasteurii* for processes of biomineralization and co-precipitation of $CaCO_3$ was reported by Whiffin et al. (2007) and Al-Thawadi (2011). This process of urease-aided $CaCO_3$ mineralization has a great potential in environmental engineering applications as well as for remediation and cementation in in situ conditions (Krajewska 2018). Bibi et al. (2018) reported indigenous *Bacillus* bacteria with biomineralization capability that could enhance soil stabilization isolated from Qatari soil. A similar report observed that *B. licheniformis* was able to precipitate calcium carbonate by ureolysis (Helmi et al. 2016). This precipitation process uses carbonate ions released during urea hydrolysis and a pH shift to highly alkaline condition. It was found that the ureolytic property of *Bacillus* sp. is high with respect to any other genus and that this might be due to their physiological ability to adapt to

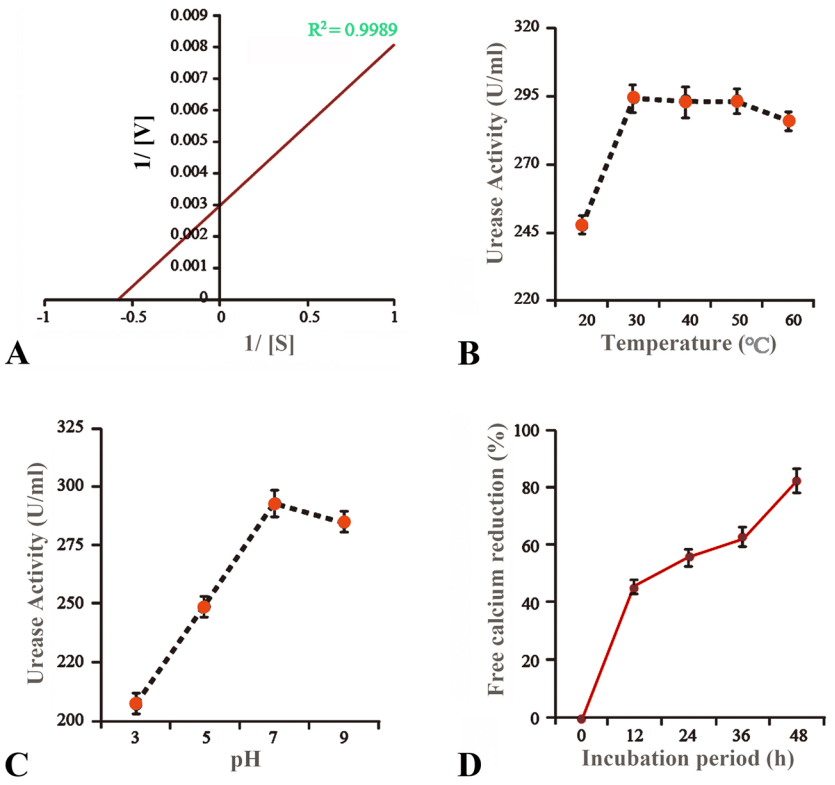

Fig. 3 Characterization of UA enzyme from *B. halodurans* isolate PO15 and evaluation of its biomineralization ability. (**a**) LB plot showing enzyme kinetics; (**b**) enzyme activity at different temperature; (**c**) enzyme activity at different pH; (**d**) Relative reduction of free Ca^{2+} in the media using urease enzyme produced by the *Bacillus halodurans* isolate PO15 [values expressed as mean ± S.D of triplicate experiment]

stressed conditions (Helmi et al. 2016). Moreover, this also facilitates bioremediation of toxic metals and radionuclides through solid-phase capture (Fujita et al. 2000).

Conclusion

This is the first report on the statistical optimization of extracellular urease production using a mangrove bacterium. A maximum urease activity of 295 U/ml was achieved by *B. halodurans* PO15 strain during statistical optimization. There was a tenfold increase in enzyme activity and the purified enzyme exhibited a high specific activity of 62.34 U/mg. The thermostable urease was active up to 60 °C and exhibited maximum activity at pH 7. UA of *B. halodurans* PO15 has potential for microbial-induced biomineralization with a reduction of free Ca^{2+} to about (82.8 ± 0.17)%. The microbial-induced calcium precipitation (MICP) using the UA enzyme could be useful in many environmental engineering applications and this opens up new avenues for simultaneous carbon mitigation and biomineralization.

Materials and methods

Isolation and screening of urease-producing bacteria

Five sediment samples were collected from different locations of the Poovar mangrove system (N. Lat. 8°18′32″ to 8°18′6″ and E. Long. 77°4′32″ to 77°5′14″), located in southern Kerala, India. The samples were collected using sterile cylindrical PVC cores with a diameter of 10 cm. The samples were stored in iceboxes and transported to the laboratory. Upon reaching the laboratory, the samples were serially diluted up to 10^{-6} in physiological saline (0.85% NaCl) and plated onto nutrient agar (NA) (Hi Media, Mumbai, India) supplemented with 5% NaCl (Sigma Aldrich, USA) for the isolation of distinct bacterial colonies. Isolated bacterial colonies were subjected to urease enzyme screening on Urea agar base agar plates. The colour change of media from orange–yellow to deep pink indicated urease production.

Bacterial culture

A positive bacterial strain with high urease enzyme activity was isolated. The strain was identified as *B. halodurans,* strain PO15, based on the 16S rRNA ribotyping (Refer Nathan et al. 2018 for details of isolation and characterization). In this study, *B. halodurans,* strain PO15 was used for

statistical optimization. The bacterium was grown in nutrient broth with 5% NaCl and incubated at 37 °C for 24 h. The culture was centrifuged at 10,000 r/min at 4 °C for 10 min to obtain crude urease enzyme.

Urease activity

The urease activity was determined by spectrophotometric assay based on the Nesslerization reaction. Briefly, 1.7 ml 10 mmol/L urea was mixed with 0.2 ml of 0.05 mol/L Tris—HCl (pH 7.0) and 20 µl of the urease. The mixture was incubated at 37 ˚C for 10 min and the reaction was stopped by adding 1.5 mol/L Trichloro acetic acid (TCA) (Sigma, USA). The reaction mixture was again incubated after adding 0.5 ml of Nessler's reagent at 37˚C for 10 min and absorbance was read at 405 nm on double beam UV–Vis spectrophotometer (SYSTRONIC MAKE, MODEL 101). One unit of urease was defined as the amount of enzyme required to release one micromole of ammonia as determined from an ammonium chloride standard curve (Kayastha et al. 1995).

Statistical optimization and model validation

For enhancing the urease activity, the optimization of culture conditions was carried out based on the Box-Behnken design, using the Design Expert 9.0 software. The response surface methodology (RSM) helped develop the mathematical models for understanding the enzyme activity on independent variables (Cui and Zhao 2012). The factors that varied during optimization were incubation period, pH, inoculation percentage and incubation temperature. Each factor was studied at two different levels (− 1 low and + 1 high) (Box and Behnken 1960). Forty-three experiments were carried out in triplicate in 250 ml Erlenmeyer flasks. Bacterial inocula were prepared with 0.5 OD McFarland Standards and added according to the inoculum percentage (3–10%). The response experimental values were derived from the mean ± S.D of three independent experiments. The Box-Behnken design (BBD) is based on a second-order polynomial equation (Eq. 2). The statistical model obtained from the experiments was validated using the optimized fermentation conditions. The enzyme assay was performed and urease activity was calculated.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC, \quad (2)$$

where Y represents the response urease activity in U/ml; A, B, and C-coded independent variables; β_1, β_2, and β_3 -linear coefficients; β_0—intercept term; $\beta_1\beta_1$, $\beta_2\beta_2$, and $\beta_3\beta_3$–quadratic coefficients; $\beta_1\beta_2$, $\beta_1\beta_3$, and $\beta_2\beta_3$–interactive coefficients.

Purification and characterization of urease

For the purification process, crude enzyme was obtained after centrifugation of the bacterial culture at 10,000 r/min at 4 °C. 80% ammonium sulphate was added to the enzyme and the pellet was dialyzed against 250 mmol/L Tris HCl buffer, pH 8.3 at 4 °C for 48 h. The lysate was purified using affinity column chromatography (Sepharose®4B-L-tyrosine-p-aminobenzene sulfonamide). The purified enzyme fraction was dried in a vacuum desiccator, resuspended in 10 mmol/L phosphate buffer (pH 7.2), which was stored at 4 °C for further experiments. The specific activity and yield (%) of urease were calculated. The enzyme–substrate interaction was further studied using the Line weaver Burk (LB) plot. K_m and V_{max} values were derived from the LB plot. The optimum pH and temperature for the maximum urease activity was also evaluated.

Calcite precipitation experiment

The ability of the bacterial isolate to sequester atmospheric CO_2 was demonstrated using the calcium precipitation assay. For this, nutrient broth was fortified with $NaHCO_3$ (25.2 mmol/L) and $CaCl_2$ (25.2 mmol/L). The relation of UA with free Ca^{2+} reduction was evaluated over a time period of 48 h. The bacterial isolate was inoculated to the medium and incubated under static conditions at (35 ± 2) °C. The samples were retrieved at 12 h interval, were subjected to free Ca^{2+} analysis using an atomic absorption spectrophotometer (AAS) (Elico, India) after centrifuging the broth at 10,000 r/min for 5 min to obtain the supernatant. Free calcium reduction (%) was expressed as mean ± S.D from the triplicate experiments performed.

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Author contributions VKN is involved in work design, execution and manuscript preparation. JV and PA involved in manuscript preparation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and human rights statement This article does not contain any studies with human participants or animals performed by any of the authors.

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