

Statistical Optimization of Urease Production by *Bacillus halodurans* PO15, a Mangrove Bacterium Isolated from Poovar Mangroves, India

Running title: Urease production using mangrove bacteria

Abstract

Mangrove ecosystem is one of the most versatile habitats for microorganisms with high potential for producing various extracellular hydrolytic enzymes. In this study, bacteria with urease activity 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^{-1}) was subjected to optimization using Box-Behnken experimental design. The studied variables included incubation period, pH, inoculation percentage and incubation temperature. Significant factors identified based on the model were incubation period, pH, incubation temperature, and inoculum percentage, to yield a 10-fold increase in UA activity (295.80 U ml^{-1}). The specific activity of purified UA enzyme was 62.34 U mg^{-1} and was found to be thermostable (active up to 60°C). UA of *B. halodurans* PO15 has potential for microbial induced bio-mineralization with a reduction of free Ca^{2+} to about $82.8\% \pm 0.17\%$. The microbe-mediated calcium carbonate precipitation (MICP) using the UA enzyme will be beneficial in the process of bio-mineralization as well as for many industrial applications.

Keywords: Urease; biomineralization; optimization; mangrove; enzymes

Introduction

Microorganisms play significant role in the engineering of the ecosystem through various biogeochemical process (Graham et al., 2016). These processes are mostly driven by the microbial enzymes. Microbial enzymes are well studied for its application in various industries, environment management as well as for serving the mankind. Urease is one such enzyme secreted by bacteria that has numerous applications. Rapidly increasing importance of application of urease enzyme has recently drawn the attention of many researchers to the need of enzyme studies. It is extensively used as a diagnostic tool to detect 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 which has tremendous application 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 et al. 2008).

Urease (EC 3.5.1.15) is a nickel-containing enzyme which catalyzes the formation of carbon dioxide and ammonia from the substrate urea (Cheng and Cord-Ruwisch 2013) resulting in an increase in pH in 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 various factors. It is induced in the presence of urea and inhibited in the presence of ammonia and nitrogen compounds (Mobley et al. 1995). Various studies have reported the isolation of indigenous ureolytic bacteria using enrichment culture from soil, ground water and cement samples (Achal and Pan 2011; 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). Their role in microbial infections was well established through studies on *Helicobacter pylori* (Mora and Arioli 2014). The urease activity of microorganisms like *Proteus mirabilis* and *Staphylococcus saprophyticus* play vital role in the urinary tract infections through struvite- carbonate-apatite urinary stone formation. Based on the urease properties on formation of carbonate apatite stones, this enzyme finds an application in the environmental engineering. Apart from the above, urease enzyme has many medical applications such as use as new drug targets for curing many diseases.

Despite their application in the fields of biomedical and environmental engineering, the production conditions of the urease enzyme were not much explored. Previous studies on the production conditions were limited to one-factor optimization (Bakhtiari et al. 2006) and have not

concluded suitable production parameters for enhancing urease activity. For Industrial application, it is important to determine the optimal conditions for enzyme production. Traditionally, factorial method was used in kinetic studies on microbially induced carbon precipitation (MICP), but the method is costly and time-consuming. Methods such as response surface methodology (RSM) in the form of Box-Behnken experimental design was 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 were laborious and results in unreliable and inaccurate results. This statistical techniques could help in designing experiments, building models, evaluating the interactive effects of variables, and ascertain the optimum conditions (Shivam et al. 2009). RSM is widely used in bioprocess technology for optimization of remarkable types of fermentation media (Desai 2008; Rishad et al. 2016; Sunitha et al. 1999;). In a study by Khodadadi and Bilsel (2015), central composite face-centered (CCF) design was used to fit a second-order model to evaluate the microbial urease efficacy in biocementation process. Optimum conditions of enzyme specific rate and urea hydrolysis was found to be significant for the process. RSM or statistical model has employed in the optimization studies 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 a higher fold of enzyme production using the microbial strains and are essential for any industrial application. 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 period, pH, inoculation percentage and incubation temperature, to achieve maximum enzyme activity. The biomineralization ability of the urease was also evaluated in the study. This forms the first report on urease optimization through statistical models.

Results and Discussion

Screening of urease producing bacteria

A total of 52 bacterial cultures were isolated from the sediment samples from Poovar Mangrove ecosystem. Among them, 21 isolates were urease positive results on urease agar. These bacterial strains were inoculated into urease broth and urease production was quantified based on spectrophotometric assay. The urease activity of these isolates ranged from 1.8 to 28 U ml⁻¹. The bacterial isolate with maximum activity was chosen for the further experiments. The strains with high urease activity were identified as *B. halodurans* through 16S rRNA ribotyping. The urease producing bacteria is found ubiquitous in nature. However, some of the common urease producing strains reported were *Helicobacter pylori*, *Proteus vulgaris*, *Staphylococcus aureus*, and

Pseudomonas aeruginosa etc. which were pathogenic or opportunistic pathogens to humans (Stabnikov et al. 2013). Apart from these, many other bacterial strains were reported with urease production that were used in microbial induced calcium precipitation (MICP). *Bacillus* sp. VS1 and *Bacillus* sp. VUK5 have been extensively used for MICP studies (Stabnikov et al. 2013). In the biocementation studies using urease producing bacteria, spore forming strains were found to be more compatible to the environment with hyper salt concentration (Bachmeier et al. 2002; Stabnikov et al. 2013). Due to this reason, optimization experiment in the present study was conducted for the *B. halodurans*, isolate PO15 alone. The major concern for environmental application is to choose an avirulent bacterial that has no adverse effect on humans or animals. Though many high ureases producing bacterium were reported, they were associated to human pathogenesis which cannot be used for any *in-situ* environmental applications. In the present study, the mangrove bacterium, *B. halodurans* strain PO15, had no virulence reported and hence employed in the optimization analysis.

Statistical Optimization for Urease Production

In the present study, most promising urease producing isolate (*B. halodurans*, isolate PO15) was selected based on urease activity. Fig. 1 depicts the schematic representation of urease production using *B. halodurans* isolate P015, based on Box-Behnken experimental design. Initial urease activity of *B. halodurans* isolate PO15 was about 28 U ml⁻¹. This value was quite higher than the urease activity reported for *Bacillus thuringiensis* N2, a marine bacterium (3.53 U ml⁻¹) (El-Bessoumy et al. 2009). In order to elucidate the effect of various factors on urease production, culture conditions were optimized using Box-Behnken experimental design. A maximum urease activity of 295.80 U ml⁻¹, a 10-fold increase from initial activity was achieved using the design (Table 1). Significant factors identified based on the model were incubation period, pH, incubation temperature, and inoculum percentage. Though aeration was reported as one major factor in enzyme production, in case of 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 (R² value of 0.9961). The Equation 2 represents the quadratic model regression equation describing the predicted model. Tirkolaei and Bilsel (2015) reported that the conditions favoring the urease production of *S. pasteurii*, amount of urea hydrolyzed, and the rate of hydrolysis inhibited the bacterial cell growth and specific hydrolysis rate of urea and vice-versa. However, in our study, we achieve a better urease production using *B. halodurans* PO15. However, it is difficult to draw a comparison between other studies as most of them report on the urea hydrolysis rate rather than urease production.

The model fitted is represented as:

UA activity (U ml^{-1}) = $355.315 + 80.2708 * \text{Incubation Period} + -35.8631 * \text{pH} + -7.31012 * \text{Temperature} + 7.14286 * \text{Inoculum \%} + 0.125 * \text{Incubation Period} * \text{pH} + -1.7125 * \text{Incubation Period} * \text{Temperature} + -4.75 * \text{Incubation Period} * \text{Inoculum \%} + 1.25 * \text{pH} * \text{Temperature} + -2.64286 * \text{pH} * \text{Inoculum \%} + 0.921429 * \text{Temperature} * \text{Inoculum \%}$ (Equation -2)

The interaction between the various medium components and factors for achieving maximum urease production was depicted through contour plots (fig 2). From the interaction between the variables, it was found that the pH of production medium is critical factor for urease production. When medium pH vs incubation period was tested, maximum urease activity of $\sim 140 \text{ U ml}^{-1}$ was achieved on the 6th day of incubation. While in case of temperature vs incubation period, it was confirmed that urease activity is at its peak after the 5th day only. Inoculum percentage vs incubation period achieved a maximum activity of 167 U ml^{-1} 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 using *A. niger* PTCC 5011 using conventional one-factor method, a maximum activity of 2.44 U ml^{-1} 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 enzyme depends on various process variables such as nutrients, pH, temperature, incubation period, inoculum level and inducer concentration (Sharma et al. 2009). Optimization of medium by the classical method involves changing one independent variable (i.e., nutrient, pH, temperature) while keeping all variables constant. However, the one-factor optimization is extremely time-consuming, expensive for a large number of variables (Okay and Rodrigues 2014), often resulting in wrong conclusions. Use of statistical-based approaches like response surface methodology (RSM) could overcome the limitations of single factor optimization process. Also, RSM based approach requires fewer trials to calculate numerous variables and their interactions, compared to other optimization methods (Managamuri et al. 2017; Peng et al. 2018).

Moreover, for urease production using *A. niger*, except orthogonal array design-based approach (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 forms the first report on statistical optimization of urease production from *B. halodurans*. From the ANOVA results, the model F-value of 6.65 and the P-values < 0.005 indicated that the model was significant (Table S1). The statistical model was validated through detection of 295 U ml^{-1} urease activity with optimized factors. Recently, El-Bessoumy et al. (2009) reported extracellular urease production from *Bacillus thuringiensis* N2 however, the enzyme activity was very low.

Purification and characterization of UA

A specific activity of 62.34 U mg⁻¹ was observed for the purified urease with 5.6 -purification fold and a yield of 87%. The specific activity of the isolate PO15 was higher than the reports on urease derived from *Aspergillus creatinolyticus* (32.74 U mg⁻¹), *Lactobacillus reuteri* (13.0 U mg⁻¹) (Kakimoto et al. 1989), *A. niger* (0.325 U mg⁻¹) (Smith et al. 1993), and *R. oryzae* (0.18 U mg⁻¹) (Geweely 2006). Based on the LB plot, a V_{max} of 333.33 mM mg⁻¹ min⁻¹ with K_m values of 1.7 mM was observed for UA. The LB plot showing the enzyme kinetics is depicted 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 the maximum production and urease activity at 35°C and the least activity was exhibited 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). Though in our study, we observed a maximum urease activity at 35°C, 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 the increase of pH from 3.0 to 9.0 (Khan et al. 2019). Some bacterial urease exhibited high activity towards alkaline conditions (pH of 9.0) (Phang et al. 2018), while some had maximum urease activity at pH 8 (Mirbod et al. 2002; Danial et al. 2015). Two fungal isolates of the genus *Aspergillus* had an optimum pH of 8.0 and pH 8.5 (Kappaun et al. 2018). In general, the fungal urease shows their maximum activity in the basic medium while bacterial urease tend to have variability (Khan et al. 2019). The higher thermostability favours the application of UA in various industrial and environmental engineering applications.

Urease mediated calcium precipitation

To understand the rate at which CO₂ is trapped as carbonates, a calcium carbonate precipitation study was carried out. The relative reduction of free calcium in the media was depicted in fig 3d. *B. halodurans* PO15 could achieve a reduction of 82.8% ± 0.17% free Ca²⁺. Maximum reduction was observed after 48 hrs incubation. For the bioremediation of CO₂ 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 CaCO₃ has been reported (Whiffin et al. 2007; Al-Thawadi 2011). This process of urease-aided CaCO₃ 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 isolated from Qatari soil that could enhance soil stabilization. Similar report was observed for *B. licheniformis* that involved to precipitate calcium carbonate by ureolysis (Helmi et al. 2016). This precipitation process make use of carbonate ions released during urea hydrolysis and of a pH shift to highly alkaline condition. It was found that ureolytic property of *Bacillus* sp. is high with respect to any other genus that might be due to their physiological properties to adapt to 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 form the first report on statistical optimization of extracellular urease production using a mangrove bacterium. A maximum urease activity of 295 U ml⁻¹ was achieved by *B. halodurans* PO15 strain during statistical optimization. There was a 10-fold increase in enzyme activity and purified enzyme exhibited a high specific activity of 62.34 U mg⁻¹. 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 bio-mineralization with a reduction of free Ca²⁺ to about 82.8% ± 0.17%. The microbe-mediated calcium carbonate precipitation (MICP) using the UA enzyme could be useful in many environmental engineering applications and this open up new avenues for simultaneous carbon mitigation and bio-mineralization.

Material and Methods

Isolation and screening of urease producing bacteria

About five sediment samples from different locations of the Poovar mangrove (N. Lat. 8°18'32" to 8°18'6" and E. Long. 77°4'32" to 77°5'14") located in the south part of Kerala, India. The sediment 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

We isolated a positive bacterial strain with high urease enzyme activity. The strain was identified as *B. halodurans*, strain PO15 based on the 16S rRNA ribotyping (Refer Nathan et al.,

2019 for details of isolation and characterization). In this study, we used this strain, *B. halodurans*, strain PO15 for statistical optimization. The bacterium was grown in nutrient broth with 5% NaCl and incubated at 37°C for 24 hrs. 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 mM urea was mixed with 0.2 ml of 0.05 M 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 M Tri chloro 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 Box-Behnken design using the Design Expert 9.0 software. The response surface methodology (RSM) will be helping in developing mathematical models for understanding the enzyme activity on independent variables (Fengjie and Liming 2012). The factor 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). A set of 43 experiments were carried out in triplicate in 250 mL Erlenmeyer flasks. Bacterial inoculum was prepared with 0.5 OD McFarland Standards and added according to the inoculum percentage (3%-10%). The averages of three readings were considered as The response experimental values were derived from mean ± S.D of three independent experiments. Box-Behnken design (BBD) is based on a second order polynomial equation 1. The statistical model obtained from the experiments were validated using the optimized fermentation conditions. The enzyme assay was performed and urease activity was calculated.

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC \text{ (Equation 1)}$$

where Y represents the response urease activity in U ml⁻¹; A, B, and C-coded independent variables; β_1 , β_2 , and β_3 -linear coefficients; β_0 - intercept term; β_{11} , β_{22} , and β_{33} - quadratic coefficients; β_{12} , β_{13} , and β_{23} - interactive coefficients

Purification and characterization of urease

For purification process, crude enzyme was obtained after centrifugation of bacterial culture at 10,000 r/min at 4°C. To the enzyme, 80% ammonium sulphate was added and the pellet was dialyzed against 250 mM Tris HCl buffer, pH-8.3 at 4°C for 48 hrs. The lysate was purified using the affinity column chromatography (Sepharose®4B-L-tyrosine-p-aminobenzene sulfonamide). The purified enzyme fraction was dried in a vacuum desiccator, resuspended in 10 mM 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. Km and Vmax 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 bacterial isolate to sequester atmospheric CO₂ was demonstrated using the calcium precipitation assay. For this, nutrient broth was fortified with NaHCO₃ (25.2 mM) and CaCl₂ (25.2 mM). The relation of UA with free Ca²⁺ reduction was evaluated over a time period of 48 hrs. The bacterial isolate was inoculated to the medium and incubated under static conditions at 35±2°C. The samples were retrieved at 12 hrs interval, were subjected to free Ca²⁺ 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.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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List of figure legends

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

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 %; and (F)- Temperature vs inoculum %

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 PO-15 [values expressed as mean \pm S.D of triplicate experiment]

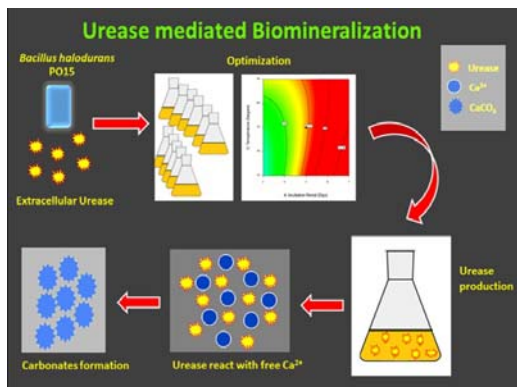


Figure 1

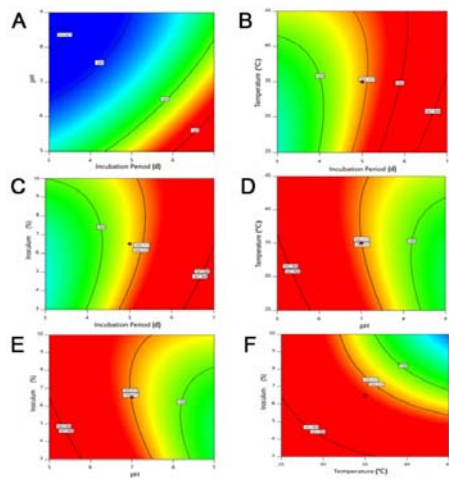
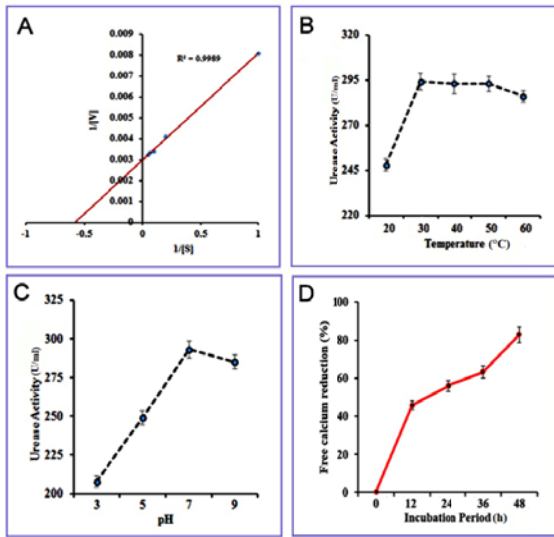


Figure 2



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Figure 3