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# Investigation of Acid-Neutralizing Property of *Bacillus cereus* GUF8

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## ABSTRACT

Extreme pHs, especially acidic pHs, are among the environmental stresses that some bacteria face. The most important strategies that bacteria employ for acid adaptation include either production of acidophilic proteins, or raising the pH of the microenvironment by secretion of basic compounds including ammonia produced by the action of urease. Considering the importance of acid-neutralizing bacteria, we previously isolated *Bacillus* sp. GUF8 from acidic soil of a tea plantation and identified it as *Bacillus cereus* using standard bacterial identification methods and 16S rDNA sequencing. Tea plantation soil is intrinsically acidic and so allows survival for only acidophilic or acid-tolerant bacteria. In the light of this fact, we investigated acid tolerance and/or adaptation property of *B. cereus* GUF8 in the present study. Our results showed that the isolate is an acid-tolerant and not acidophilic strain which neutralizes the acidic microenvironment by secreting urease. Urease production increases until the pH reaches to about 8.0-9.0, whilst the isolate grows optimally at around pHs 6.0-7.0 and there is a lag period of about two days prior to active growth on media at pH 4.0. The results suggest that the isolate has potential for use as a neutralizing agent in acidified environments.

**Keywords:** *Bacillus cereus* GUF8, Acid-neutralizing, Urease, Acid tolerant

## INTRODUCTION

Bacteria face various environmental stresses such as pH changes during their life and their survival in these environments depends on their adaptation ability to abiotic stresses. Low pH is an important stress that microorganisms may face; so, adaptation to and survival in such environments is very essential for them. On the other hand, the acid and alkaline balance in an organism plays an important role for its survival and proper functioning [1]. Bacteria inhibit their acidic environments by producing acidophilic proteins and enzymes, and or increasing the pH of the microenvironment at first and then producing acid-tolerant enzymes that are active at pH values near-neutral. In the latter case, some bacteria produce urease, which ultimately converts urea into ammonia ($NH_3$) and carbon dioxide ($CO_2$), and so the pH of the microenvironment increases [2,3].

Urease (EC 3.5.1.5) is a nickel-containing metalloenzyme that breaks down one molecule of urea into one molecule of carbon dioxide and two molecules of ammonia. Urease is produced by many organisms, including some bacteria, fungi, plants, and even some invertebrates and there are significant amino acid similarities between all known ureases [3,4]. The important role of bacterial ureases in the pathogenesis of a number of species has been proven, including *Staphylococcus saprophiticus*, *Ureaplasma urealiticum*, *Proteus mirabilis, Yersinia enterocolitica* and others [3-5]. Urease from *Helicobacter pylori* is one of the most frequently mentioned examples in recent years because of its critical role in bacterial pathogenesis and the high prevalence of this human pathogen [5,6]. There are several available assays for measuring urease activity that are mainly based on the measurement of the amounts of products released during the reaction.

It has been proposed that acid-neutralizing microbes can

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be used to remedy the effects of environmental acidification and also some of them are beneficially used in the dairy industry [7,8]. Despite the importance of the issue, not much is known about the mechanisms of acid tolerance and/or adaptation and there is great emphasis on the identification of new acid-neutralizing bacteria and investigation of their acid tolerance and/or adaptation mechanisms. In our previous studies, we characterized an α-amylase (with high activity in a broad temperature range) from a new acid-neutralizing *B. cereus* species (*B. cereus* GUF8). The bacterium was isolated from soil samples of a tea plantation located in the north of Iran (Fooman, Guilan, Iran) [9,10]. The optimum pH for enzyme activity was pH 6.0 and approximately 75 and 50% of its maximal activity was retained at pHs 8.0 and 9.0, respectively. Tea plantation soil is intrinsically acidic (pH 4.5), thus providing a suitable habitat for acidophilic or acid-tolerant microorganisms. In order to find an acidophilic α-amylase producing strain, we isolated acid-tolerant or acidophilic bacteria on selective media. According to the results, *B. cereus* GUF8 exhibited the highest level of amylase activity compared to other bacteria when soil samples were cultured on starch agar plates at pH 4.0 [10]. The optimal growth of the isolate was at around neutral pH values and it exhibited delayed growth on acidic media. Considering the importance of acid-neutralizing bacteria in health, food technology and safety, and also their remedial effects on environmental acidification and a great emphasis on the identification of new acid-neutralizing bacteria [7,8,13], we decided to investigate acid tolerance and/or adaptation property of *B. cereus* GUF8 in the present study.

## METHODS

### Chemicals

Tris, NADH, α-ketoglutarate, and glutamate dehydrogenase were obtained from Sigma (St. Louis, MO. USA). PCR reagents were purchased from Boehringer Mannheim (Boehringer Mannheim co., Mannheim, Germany). Microbiological media and all other chemicals were obtained from Merck (Merck, Darmstadt, Germany).

### Isolation and Identification of Microorganisms

Soil samples were collected from a tea plantation located in the north of Iran (Fooman, Guilan, Iran). 20 grams soil samples were added to 100 ml sterile water in an Erlenmeyer flask. 1 ml of this suspension was then added to 9 ml of sterile distilled water and a serial dilution ($10^{-1.0}$-$10^{-9.0}$) was prepared, while the content was agitated. About 1 ml of each dilution was added and distributed on an isolation medium containing nutrient agar supplemented with 1% yeast extract and 2% agar, and the pH adjusted to 4.0. After incubation at 37 °C, the growing colonies were isolated on fresh plates. The isolate growing in these conditions was investigated for physiological, morphological and biochemical characteristics with reference to Bergey's manual of systematic bacteriology [11]. Molecular identification based on 16S rDNA sequencing was carried out as described previously [10].

### Measurement of pH Changes During Growth

Preliminary experiments were carried out by adding phenol red to nutrient agar plates with an initial pH set at 5.0 and changes in the medium color were noted. The changes in pH in liquid media were also studied by inoculating nutrient broth set at initial pH values (from 4.0 to 9.0), taking samples at regular intervals and measuring the pH. Ammonium production from urea was determined by inoculating the isolate onto urea agar and observing the changes in color.

### Urease Aassay

Urease activity was determined based on a coupled assay following glutamate dehydrogenase-mediated NADH oxidation that was coupled to ammonia oxidation [12]. Glutamate dehydrogenase 12 U $ml^{-1}$ was incubated with 250 μM NADH for 5 min. α-ketoglutarate (1 mM), urea (10 mM) and culture supernatants from the media were added and the decrease in absorbance at 340 nm was followed. One unit of the enzyme activity was defined as the amount of the enzyme that hydrolyzes 1 μmol of the substrate (urea) per min.

## RESULTS AND DISCUSSION

Bacteria face various environmental stresses, such as pH changes and their survival in stressful environments depends on their adaptation ability to abiotic stresses. Soil

acidity is one of the important stresses that bacteria may encounter; therefore, adaptation to/and survival in such environments is very essential for their survival. Acid adaptation is not only a fascinating biological problem, but also helps in our understanding of pathogenic and environmentally important microorganisms [1,3]. Beneficial microorganisms used in the food and especially the dairy industry (*e.g*. *Lactococcus lactis* and *Lactobacillus acidophilus*) face acid stress during fermentation processes and possess mechanisms of acid tolerance [13]. Similarly, probiotic bacteria have to survive in acidic conditions of stomach until they reach their destination in the intestine and play their beneficial roles there. On the other hand, adaptation and survival at acidic pHs are important factors in the pathogenicity of gastrointestinal bacteria and also foodborne pathogenic bacteria, such as *Escherichia coli*, *H. pylori*, *Salmonella typhimurium*, *Aeromonas hydrophila*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, and *Enterococcus faecalis*, as well as the oral cariogenic organism *Streptococcus mutans*. These are of great concern in food safety and health [1,3,5,13].

Microorganisms which possess heightened resistance to low pH and are capable of neutralizing acids, are also important for remedying the effects of environmental acidification [7,8]. Excess use of fossil fuel has led to acid rain and this has in turn led to many serious problems in different countries. One of the serious consequences of this acidification is that cations required for plant growth are leached out in soil acidification, and so vegetation and forests are seriously damaged. This in turn leads to more flooding and all the other problems which ensue. Development of microbial methods to neutralize acidified water and measurement of the neutralization activities of various strains of microorganisms have in recent years attracted particular consideration [8].

Considering the importance of acid-neutralizing bacteria in health and also food technology and safety, on the one hand, and their abilities to remedy the effects of environmental acidification on the other hand, there is a great emphasis on the identification of new acid-neutralizing bacteria and investigation of their acid tolerance and/or adaptation mechanisms. In the present study, along with the efforts of various research groups trying to find new acid-neutralizing bacteria, we isolated a new acid-neutralizing *B. cereus* species from soil of a tea plantation located in Fooman, Guilan province of Iran; and investigated its acid tolerance and/or adaptation property.

## Isolation of an Acid-tolerant Bacterium

Following the initial incubation of the soil samples on agar plates (pH 4.0), *B. cereus* GUF8 was selected for further investigations. There was a delay of about 48 h before active growth on the acidic medium (pH 4.0), suggesting the bacterium may be affecting changes in its microenvironment. The results of the bacterium incubation on the same medium but with a higher pH value (pHs 5.0, 6.0 and 7.0) revealed that the isolate grew faster at pH values close to neutral. Addition of phenol red to the starch medium, with an initial pH of 5.0 showed that the pH of the medium is constantly raised to about pH 7.0 during the course of bacterial growth (Fig. 1A).

The important role of microorganisms possessing a high resistance to low pHs and a high acid neutralizing ability, has been proven to remedy the effects of environmental acidification. Nabeshima *et al*. developed a microbial method for neutralization of acidified water and measured the neutralization activities of various strains of bacteria, fungi and yeasts with an acidic medium (pH 3.0) [8]. According to their results, fungi showed high neutralization properties, and *Rhizopus delemar* neutralized acidic media by secreting basic compounds, including ammonia. *R. delemar* raised the pH value of pond water from acidic to neutral pH values (4.0 to 7.0) within 2 days. At present, various studies are being carried out on use of bacteria in bioremediation of environments acidified by human activity [7]. A comparison of the results obtained in the present study and that obtained for *R. delemar* [8] indicates that *B. cereus* GUF8 can neutralize acidic environments even faster than fungi. This topic needs further examination.

## Identification of the Strain

*Bacillus* sp. GUF8, was identified as *Bacillus cereus* based on standard bacterial identification methods and 16S rDNA sequencing [10].

## Optimal pH for Bacterial Growth

The isolate was grown in nutrient broth media with different pH values adjusted to those shown in Fig. 2 and

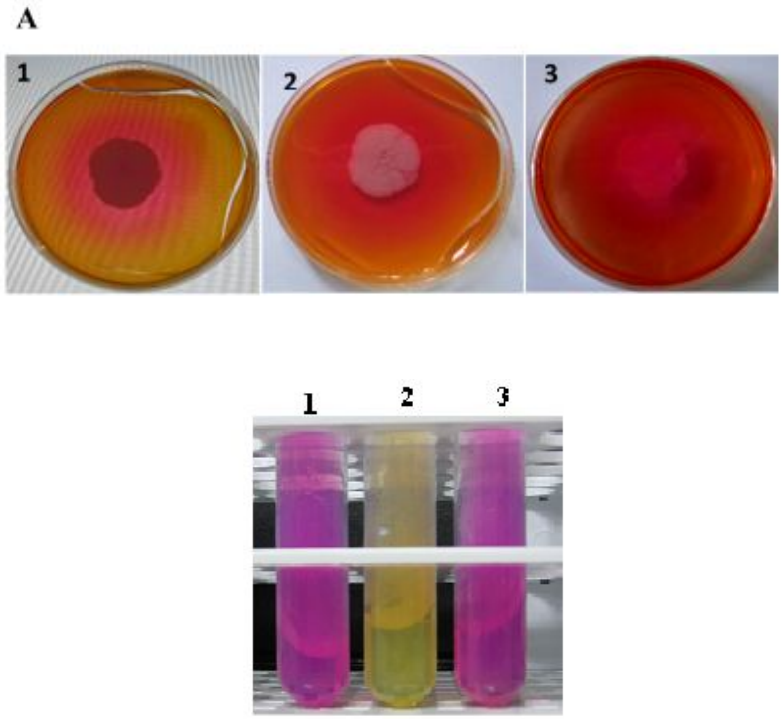


**Fig. 1.** (A) Incubation of *B. cereus* GUF8 on nutrient agar in the presence of 0.002% phenol red on an initial pH of 5.0. Plate 1: 16 h post incubation (pi); plate 2: 24 h pi and plate 3: 36 h pi. (B) Urease test, 1: *Klebsiella* as a positive control, 2: *Bacillus subtilis* as a negative control, 3: *B. cereus* GUF8.

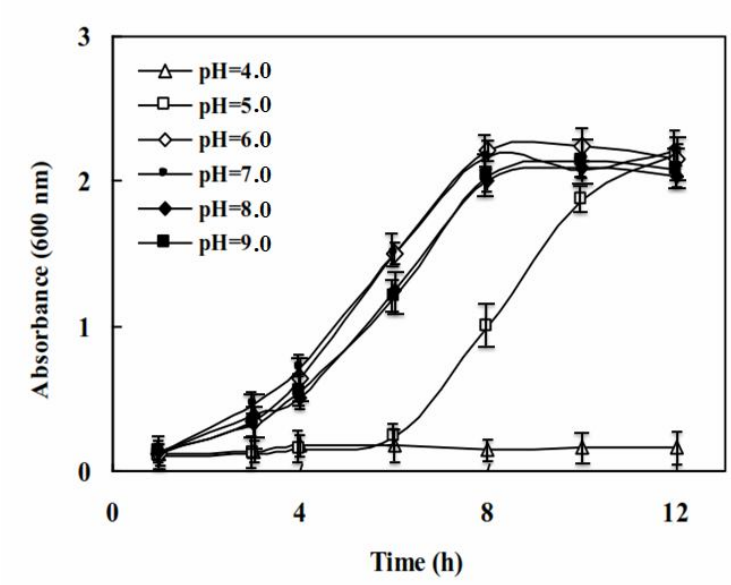


**Fig. 2.** Growth of *B. cereus* GUF8 on nutrient broth with the initial pH adjusted to between 4.0 to 9.0. The isolate shows optimal growth at pH 6.0-7.0.

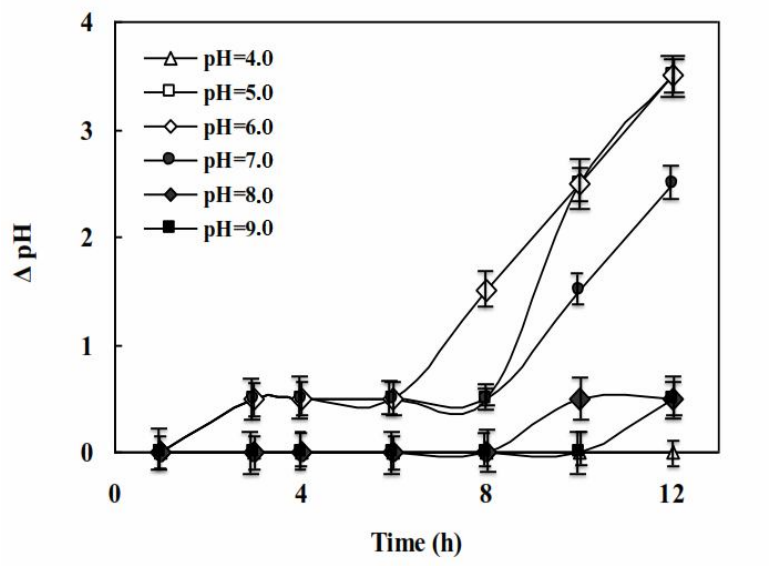


**Fig. 3.** pH changes during bacterial growth on nutrient broth with different initial pH values.

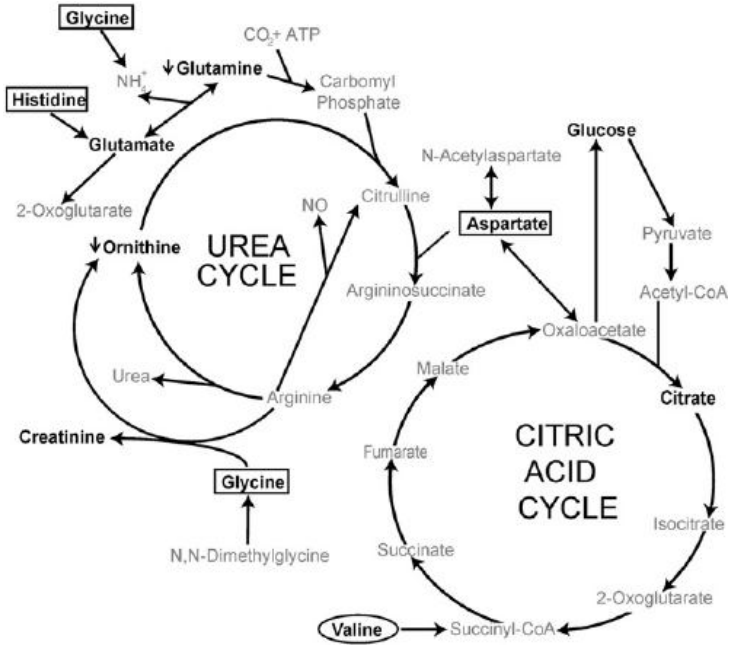


**Fig. 4.** Metabolic scheme for production of urea.

the absorbance at 600 nm was read at regular intervals. The isolate showed optimal growth at pHs 6.0-7.0. There is no growth at pH 4.0 in the first 12 h. Whilst cultures at pH 7.0 and 8.0 do not show significant change, the most increase in the medium pH is seen at pH 5.0 (Fig. 3). pH measurements during the growth period showed that the pH of the medium is raised to near neutrality by bacterial activity.

Acid-neutralizing strains E25$^T$ and E21, belonging to the

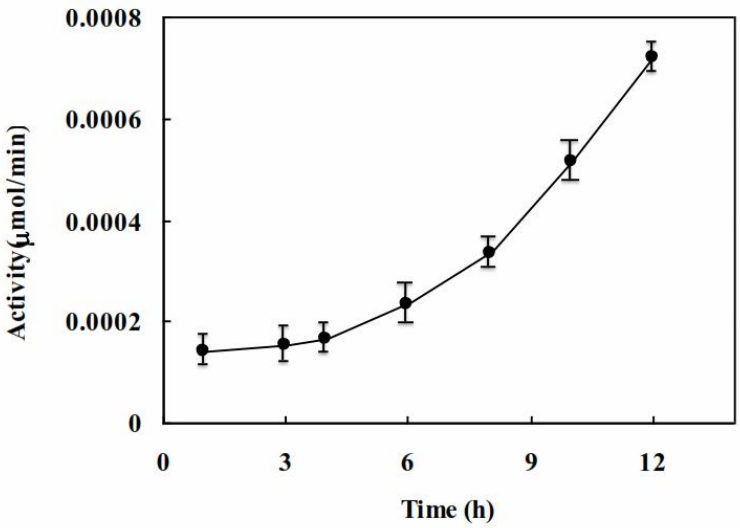


**Fig. 5.** Measurement of urease activity during the first 12 h pi in nutrient broth with an initial pH of 5.0.

genus *Burkholderia*, were isolated from torpedo grass (*Panicum repens*) that grows in highly acidic swamps (pH 2.0-4.0) in acidic regions of Thailand [14]. According to the results obtained from biochemical and physiological tests, these strains represent a novel species named *Burkholderia bannensis* sp. nov. Aizawa *et al*., and Kimoto *et al*., also isolated some waterweeds-associated acid-neutralizing bacteria and reported several novel acid-neutralizing bacterial species adapted to highly acidic aquatic environments (pH 2.0-4.0) in South-East Asia [14-16].

## Measurement of Urease Activity

Incubation of *B. cereus* GUF8 on urease agar plates revealed that the bacterium secrets urease as indicated by urease assay and the change in color from yellow to pink (Fig. 1B). Enzyme assays were carried out in triplicate using a coupled assay following NADH-mediated glutamate dehydrogenase oxidation that was coupled to ammonia oxidation. Activity measurements were made of the culture supernatants from media with a starting pH of 5.0. It may be noted here that in nature urea is present both in soil samples due to excretion from animals [17] and also can be produced intracellularly by the urea cycle (Fig. 4).

The results shown in Fig. 5 exhibit an increase in urease activity from about 4 h post incubation (pi) to 12 h pi. It seems that low pH values induce urease production (Fig. 1B) until the pH is raised to near neutrality and therefore the isolate overcomes its acidic environment. Subsequently, the bacterium proceeds to grow exponentially at higher pH values (pHs 6.0-7.0). In some bacteria, including *H. pylori, Yersinia enterocolitica* and *Streptococcus salivarius*, there are some reports on a dual role for urease, in nitrogen metabolism and in acid resistance [18-20]. Presence of the urease gene in *Bacillus cereus* has been previously demonstrated [6,17], although the corresponding enzyme from this species has not been studied well and it seems to be mainly involved in nitrogen metabolism, rather than acid shock survival. Mols *et al*. reported that none of their isolated ureolytic *B. cereus* strains displayed enhanced fitness under acidic conditions or revealed increased acid stress survival in the presence of urea. In contrast, *B. cereus* GUF8 employs ureolytic activity to increase its survival capacity under acid shock conditions. Another notable point is that in Gram positive bacteria urease decomposes urea into ammonia and carbon dioxide within the cell and then the chemicals diffuse through the cell wall into the surrounding media. It is very interesting that *B. cereus* GUF8, however, secrets urease into the surrounding media and the enzyme activity was measured in the culture supernatants from bacterial media.

It is clear that evolution of urease production by bacteria to counter the effects of acidic environments is of

importance. These strains raise the pH of their microenvironment by production of basic compounds and then produce acid-tolerant enzymes that are active at pH values near-neutral. This mechanism is seen *e.g.* in *Klebsiella aerogenes* [21], *Bacillus pasteurii* [5] and *H. pylori* [19]. In some pathogenic bacteria acid-neutralization is an essential virulence factor, as shown by *H. pylori* and *Proteus mirabilis* [5]. Some other bacteria inhabiting acidic environments are intrinsically acidophilic and the proteins produced and secreted are optimally active at the extracellular pH values *e.g. Thiobacillus ferroxidans* [22].

## CONCLUSIONS

Considering the importance of acid-neutralizing bacteria in health, food technology and safety, and also the remedial effects of environmental acidification, isolation and identification of new acid-neutralizing bacteria and investigation of their acid tolerance and/or adaptation mechanisms are very important. Bacteria inhabiting acidic environments employ different strategies to overcome acidic stress. Some of them produce acidophilic enzymes and proteins, and others increase the pH of their microenvironment and then produce acid-tolerant proteins and enzymes. Since tea plantation soil is intrinsically acidic, it provides a suitable habitat for acidophilic or acid-tolerant microorganisms. In conclusion, our results showed that *B. cereus* GUF8 is an acid-neutralizing strain which overcomes its acidic environment primarily by secreting urease. Low pH values induce urease production until the pH is raised to near neutrality and then the isolate grows optimally at higher pH values. In other words, this acid-neutralizing *Bacillus cereus* strain is an acid-tolerant and not acidophilic species that neutralizes its acidic microenvironment by secreting urease which subsequently produces ammonia. Therefore it improves conditions for its growth and survival in acidic soil of the tea plantation.

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