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SHORT COMMUNICATION

Biochemical study of trehalase activity in *Aspergillus niger* conidia

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ABSTRACT

Trehalase (α, α-trehalose-1-C-glucohydrolase, EC 3.2.1.28) is a glycoside hydrolase enzyme that catalyzes the conversion of trehalose to glucose. Trehalose hydrolysis by trehalase enzyme is an important physiological process for various organisms, such as fungal spore germination, insect flight, and the resumption of growth in resting cells. So by studying biochemical properties of trehalase we can control the spore germination of harmful fungus, *Apergillus niger*. Till now trehalase has been reported from many other organisms including plants and animals, Trehalase has been purified and characterized from various organisms such as *Saccharomyces cerevisiae*, *Lentinula edodes*, *Acidobacterium capsulatum*, and *Medicago sativa*, as well as root nodules of *Phaseolus vulgaris*, soybean and from the from the seeds of *Triticum aestivum. In this study*, trehalase was purified from *Aspergillus niger* grown on cellulosic medium. And this purified trehalase was tested for various requirements it may need for enzymatic activity such as inorganic phosphate and Magnesium ions (Mg^{2+}).

***Keywords*:** *Aspergillus niger*, Conidia, Enzyme Activity, Trehalase

1. INTRODUCTION

Trehalase is a glycoside hydrolase enzyme that catalyzes the conversion of trehalose to glucose. Trehalose hydrolysis by trehalase enzyme is an important physiological process for various organisms, such as fungal spore germination, insect flight, and the resumption of growth in resting cells. So in present study we are concerned about its role in spore germination in fungal cells of *Aspergillus niger*.

Aspergillus niger is a dangerous airborne fungal pathogen. *Aspergillus niger* contains toxins that can effect people with weak immune systems and can make them sick and can sometimes lead to death. These toxins are most commonly inhaled by people who work around plants or peat, and can cause a lung disease called Aspergillosis, which has infected over 300,000 people worldwide (Keir *et al*., 2014). *Aspergillus niger* secretes several toxins, such as malformin C, and ochratoxin A which are responsible for food spoilage (Pitt & Hocking, 2009; Geiser *et al.*, 2006). Food spoilage because of fungi is of great concern because of economical losses, food supply shortage and its effects on human and animal health. It is difficult to evaluate the intensity of this problem, but it is estimated that 25 % of all agricultural products are contaminated with these mycotoxins (Dagnas & Membre, 2013). *Aspergillus niger* also causes a disease called black mould on certain fruits and vegetables such as grapes, apricots, onions, and peanuts, and is a common contaminant of ornamental plants. Infection of onion seedlings by *Aspergillus niger* can become systemic, manifesting only in conducive conditions. It causes common postharvest disease of onions, in which the black conidia can be observed between the scales of onion bulb. Mould growth causes quality loss in food such as bad smell and bad flavours, discoloration, visible mould growth, and loss of structure and texture. Main problem is the production of mycotoxins during mould growth, which makes the food toxic and unhealthy (Dagnas & Membre, 2013; Filtenborg *et al.*, 1996). Mycotoxin production is linked to fungal development mainly to sporulation. Some of these substances induce spore-formation, others act as virulence factors or increase the fungal strain competitiveness (Calvo *et al.*, 2002).

Mycotoxins are mainly secreted in food with high water content and quickly diffuse and contaminate the whole foodstuff. Mycotoxins are mainly resistant to different types of chemical and physical treatments (Filtenborg *et al.*, 1996). The known toxic effects of mycotoxins are diverse, the most common are immune system suppression, carcinogenicity, and their toxic for liver, kidney and reproductive system. (Creppy, 2002; Filtenborg *et al.*, 1996) Several species of *Aspergillus* are mycotoxin producers. The effects on animals include a decrease in antibody responses, a size reduction in immune organs, and an alteration in the production of cytokines. Food that has been contaminated by *Aspergillus niger's* toxic metabolite majorly affects poultry industry.

So by studying the activity of trehalase enzyme, one may learn to control the germination by controlling trehalase activity and thus prevent food spoilage. This work may contribute to food industry by increasing shelf-life of food. And this enzyme study may also be helpful in controlling infections, which are caused by fungal spores dispersed trough air such as Aspergillosis.

2. MATERIALS AND METHODS

Microorganism and culture conditions

Aspergillus niger NS2 was maintained on 50ml media slants [4% (w/v) cellulosic media and 1.8% (w/v) agar] 28 °C for 10 days then shifted to 4 °C for 5 days. All routine chemicals used in present study were of analytical grade.

Harvesting of conidia

Conidia from 15-day old culture of *Aspergillus niger* were harvested with chilled sterile distilled water by gently scraping the surface of culture with a sterilized glass rod. This conidial suspension was centrifuged at 12,000 x g for 10 min. The conidial pellet was freezed and then lypholysed.

Purification of Trehalase from *Aspergillus niger*

To 5 g spore of conidia , 10 g of alumina was added and cell suspension was prepared using 0.01 M sodium citrate buffer containing 1 mM phenylmethanesulfonyl fluoride. This suspension was ruptured using glass beads. The homogenate was centrifuged and resultant paste was suspended in 200 ml of 0.01 M sodium acetate buffer and was centrifuged. To the supernatant solution, 1 M acetic acid was added and the pH was adjusted to 4.0. The precipitate formed was removed by centrifugation. The supernatant was cooled to 0 °C, and equal amount of cold acetone was added. The precipitate was collected and dissolved in 10 ml of sodium acetate buffer, and dialyzed against 0.01 M phosphate buffer. The resultant solution adsorbed on Diethylaminoethyl (DEAE)-cellulose column which had been equilibrated with 0.01 M phosphate buffer. The enzyme was eluted by applying gradient of 0.05 to 0.3 M NaCl. All purification steps were carried out at 4 °C. At each step of purification, trehalase activity and protein content were determined using Bradford method (Bradford, 1976).

Assay of trehalase

Trehalase activity was assayed using 3,5-Dinitrosalicylic acid method by Miller (Miller, 1959). The reaction mixture contained 100 mM trehalose, 50 mM sodium acetate buffer (pH 5.0), and 0.25 ml enzyme extract in a final volume of 0.5 ml. One unit is defined as the amount of trehalase that produces 1 nmol of glucose in 1 min at 37 °C.

Effect of inorganic phosphate and arsenate on trehalase activity

To study the effect of inorganic phosphate and arsenate on trehalase activity, 0.5 ml of incubations contained 0.3 ml of trehalose, 5 mM $MgCl_2$, 10 units of purified protein and various amounts of sodium phosphate and sodium arsenate and final volume made up with 50 mM sodium acetate buffer (pH 5.0).

Effect of Magnesium ions (Mg^{2+}) on trehalase activity

To study the effect of Magnesium ions, 0.5 ml of reaction assay was used, containing 0.3 ml of 0.1 M trehalose, 10 units of purified protein, increasing amounts of $MgCl_2$, and finally volume was made up with 50 mM sodium acetate buffer (pH 5.0). After incubation of 10 min, reactions were stopped by heating and amount of glucose produced was measured.

Effect of different phosphorylated compounds on trehalase activity

To study the effect of phosphorylated compounds, 0.5 ml of reaction assay was used, containing 0.3 ml of 0.1 M trehalose, 5 mM $MgCl_2$, 10 units of purified protein, varying amounts of 2 mM to 16 mM Sodium pyrophosphate, and finally volume was made up with 50 mM sodium acetate buffer (pH 5.0). After incubation of 10 min, reactions were stopped by heating and amount of glucose produced was measured.

Statistical analysis

The statistical analysis of data was done using paired Student's t-test. P-value ≤ 0.05 were considered significant.

3. RESULTS

Effect of inorganic phosphate and arsenate on trehalase activity

The enzyme activity of purified *Aspergillus niger* trehalase extracted after DEAE-cellulose chromatography was studied to demonstrate the requirement of inorganic phosphate, and optimum activity was observed at the concentration of 110 mM as seen in Figure 1.

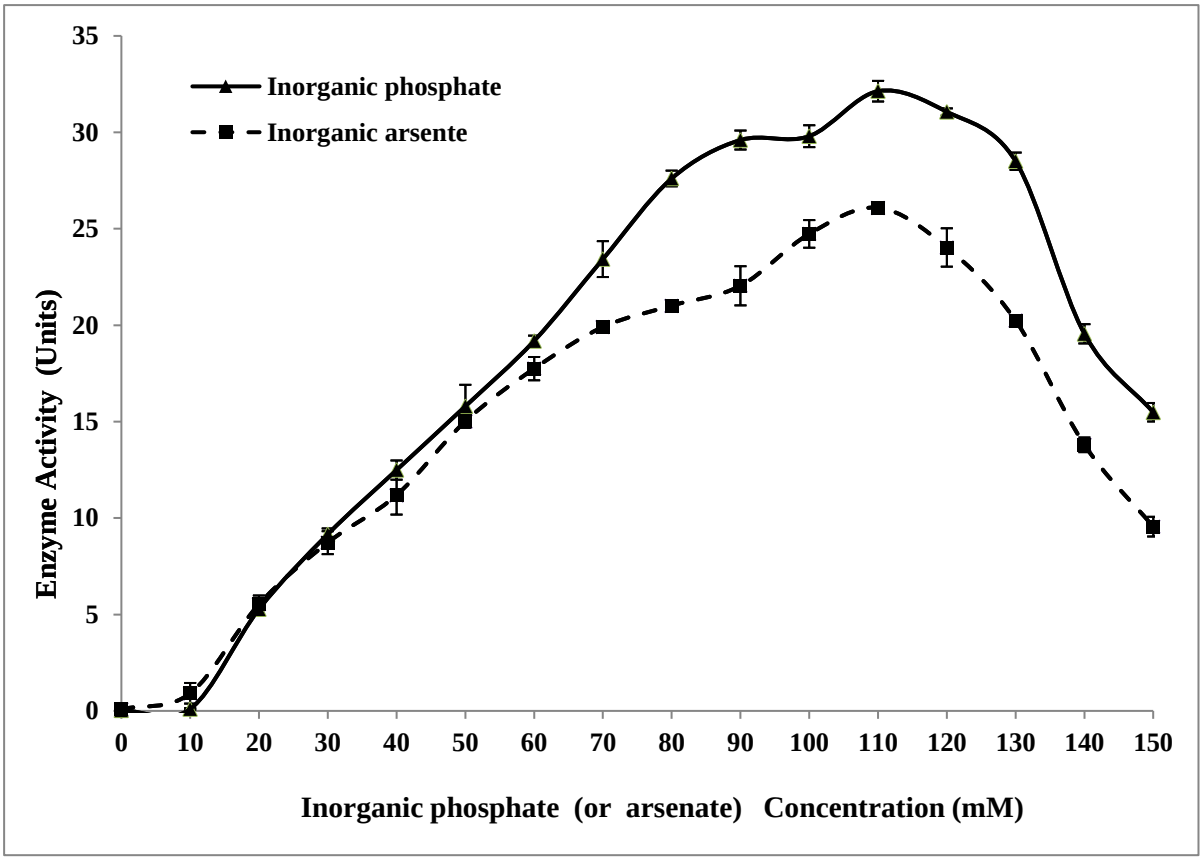

Figure 1. Effect of inorganic phosphate and arsenate on the activity of *A. niger* trehalase. Incubations contained 0.3 ml of trehalose, 5 mM $MgCl_2$,10 units of purified protein and various amounts of sodium phosphate and sodium arsenate and final volume made up to 0.5

ml with 50 mM sodium acetate buffer. After incubation of 10 min at 37 °C, reactions were stopped by heating in a boiling water bath for 5mins and the amount of glucose produced was determined by protocol described in materials and methods section. Data is the mean of three independent experiments ± standard deviation, indicated by bars, $p \leq 0.001$.

This activity profile showed that arsenate can fulfill the requirement of phosphate to some extent, but is not as much as effective as phosphate. But its activity profile at various concentrations was same as that of phosphate activity profile, this indicates that it has same effect on enzyme. At equal concentration of phosphate and arsenate (110 mM), the production of glucose was decreased by 20 % in presence of arsenate.

Effect of Magnesium ions (Mg^{2+}) on trehalase activity

The enzyme was further tested to check the requirement for Mg^{2+}, with optimum enzyme activity at concentration of 5 mM. Enzyme activity profile in presence of Mg^{2+} shown in Figure 2 suggested that magnesium ions are mainly required for trehalase activity.

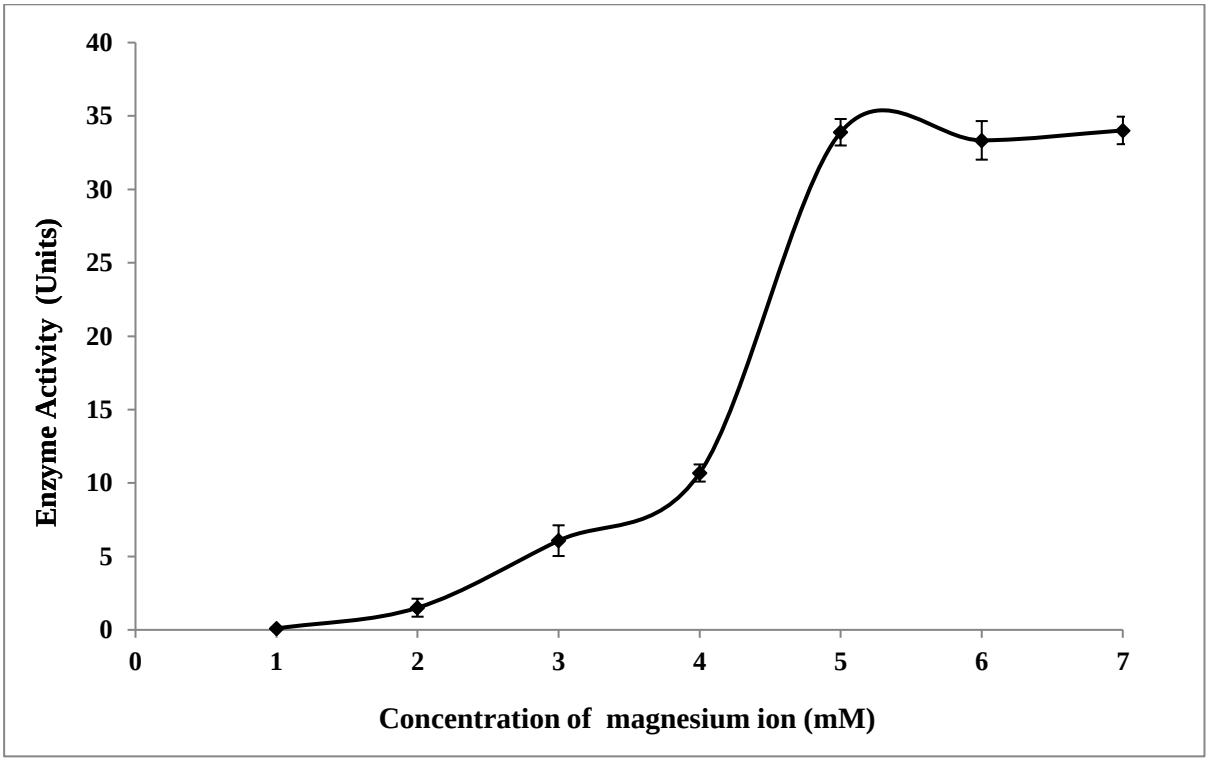

Figure 2: Effect of Mg^{2+} concentration on the activity of A. niger trehalase. Incubation mixtures contained 0.3 ml of trehalose, 10 units of purified protein and increasing amounts of $MgCl_2$ and final volume made up to 0.5 ml with 50 mM sodium acetate buffer. After incubation of 15 min, reactions were stopped by heating and glucose released was determined by method described in materials and methods section. Data is the mean of three independent experiments ± standard deviation, indicated by bars, $p \leq 0.005$**.**

Effect of different phosphorylated compounds on trehalase activity

Trehalase activity was decreased in presence of pyrophosphate and polyphosphates. Figure 3 represents the enzyme activity profile in presence of increasing concentrations of potassium pyrophosphate on the hydrolysis of trehalose into glucose at different concentrations of phosphate buffer.

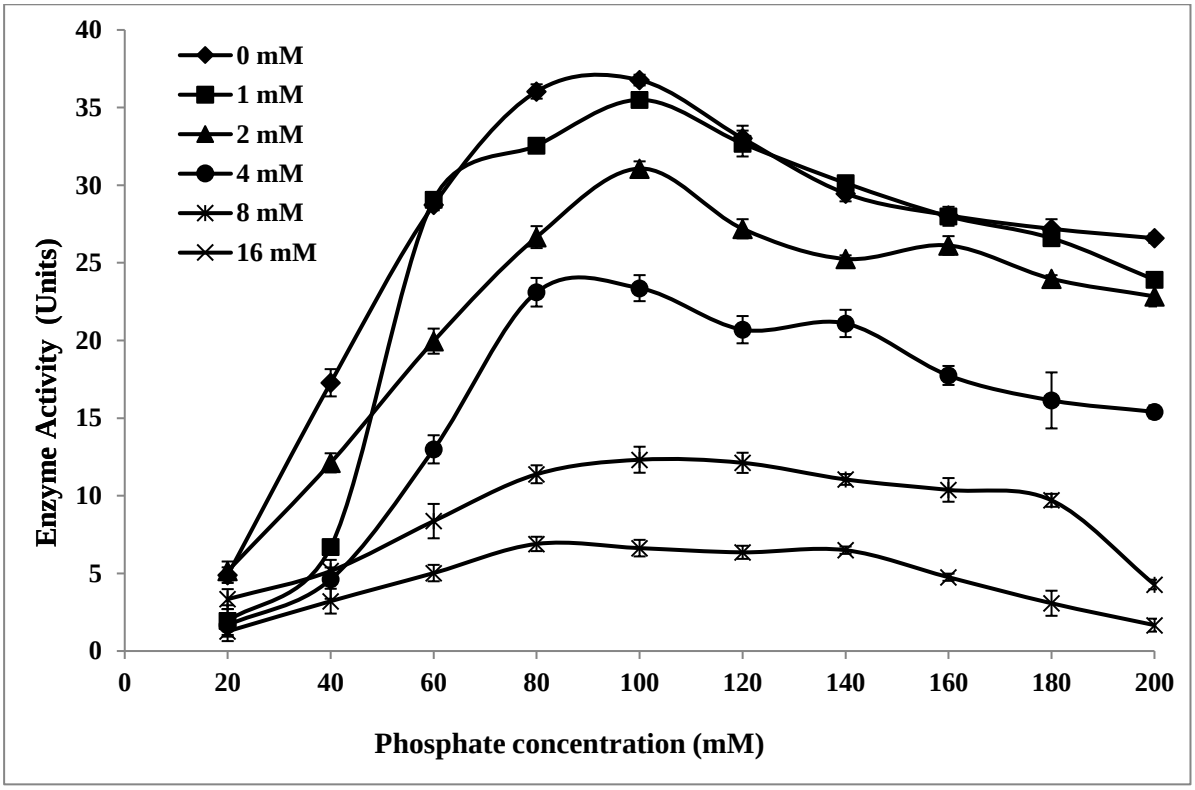

Figure 3. Inhibition of trehalase activity by sodium pyrophosphate. Reaction mixtures contained varying concentration of sodium phosphate buffer (0 to 200 mM), 6 mM $MgCl_2$, 50 mM Trehalose, 10 units of purified trehalase, and varying amounts of sodium pyrophosphate. After incubation of 10 min, reactions were stopped by heating and amount of glucose released was measured. Data is the mean of three independent experiments ± standard deviation, indicated by bars, $p \leq 0.001$

Experiments showed that in presence of 1 mM pyrophosphate, there was little effect on trehalase activity, even at lower concentrations of phosphate buffer. Further on increasing pyrophosphate concentration, there was increase in inhibition of trehalase activity. At 4 mM concentration of pyrophosphate, there was approximately 50 % of inhibition in trehalase activity. On increasing concentration there was increase in concentration. At 16 mM there was about 100 % loss of enzyme activity, even at higher concentrations of phosphate.

Polyphosphates also showed inhibition in trehalase activity as shown in Figure 4. Four polyphosphates were tested AMP, ADP, ATP and UTP.

AMP caused about 30 % of inhibition at 20 mM, whereas ADP caused approximately 50 % inhibition at same concentration and ATP inhibited enzyme activity by 60 %. UTP showed equal inhibition at lesser concentration of 10 mM.

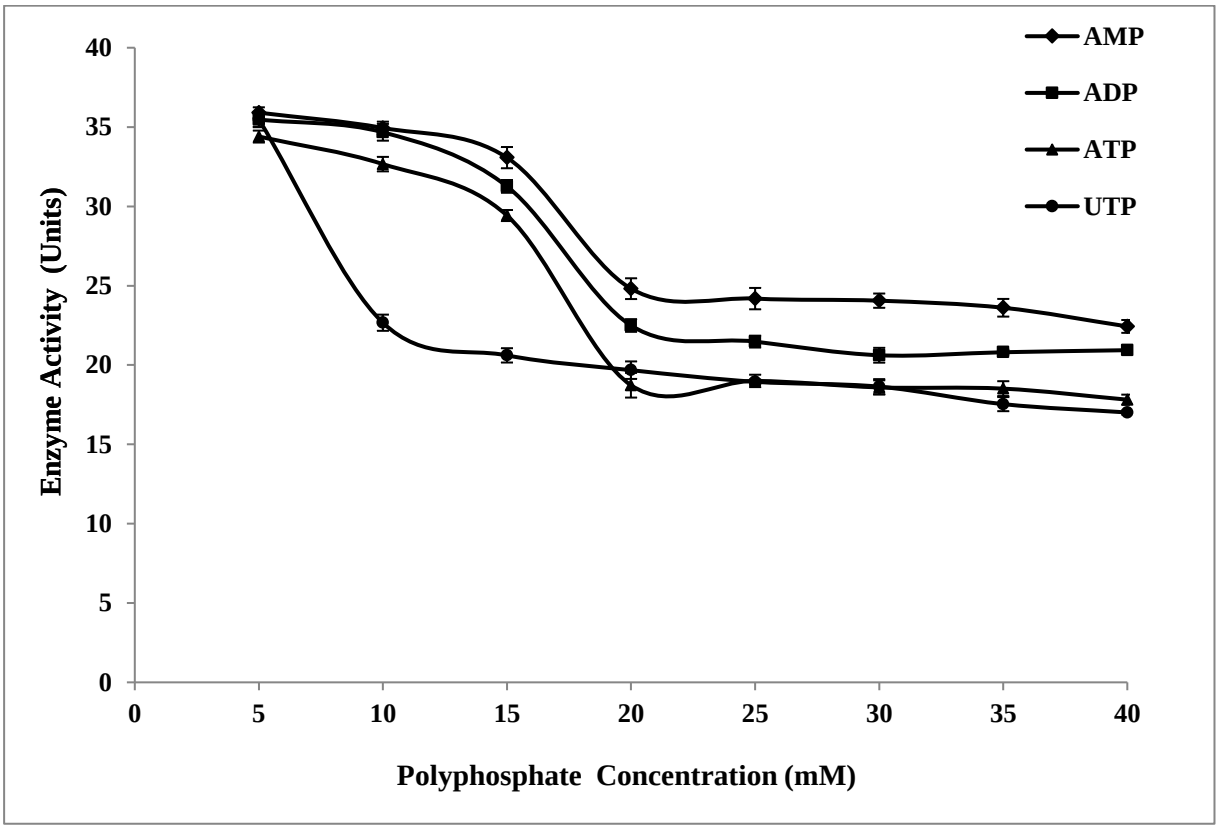

Figure 4. Inhibition of trehalase activity by polyphosphates. Reaction mixtures contained varying concentration of sodium phosphate buffer, 6 mM $MgCl_2$, 50 mM Trehalose, 10 units of purified trehalase, and varying amounts of polyphosphates as shown in figure. After incubation of 10 min, reactions were stopped by heating and amount of glucose released was measured. Data is the mean of three independent experiments ± standard deviation, indicated by bars, $p \leq 0.001$.

4. CONCLUSION

The purified trehalase studied in this report required inorganic phosphate and $MgCl_2$ for enzyme activity. Incubation of this purified trehalase with other buffers resulted in phenomenal loss of enzyme activity. On further adding inorganic phosphate and $MgCl_2$ there was rise in enzyme activity. This suggests that the trehalase is activated and stabilized by phosphate and magnesium ions. Inhibition of trehalase by polyphosphates suggests that this regulates trehalose metabolism and hence spore germination. In various conditions polyphosphates protect cells from adverse conditions, but mechanism is not known. We can elucidate this study further to study the reason. It is possible that protective effect of polyphosphates is because of trehalase inhibition.

So we can strategize a technique to inhibit trehalase activity and thus prevent spore germination. And this could help us to solve various problems such as economical issue related to food spoilage and serious health concerns.

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