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Screening of Chitinase Producing Strain and Effect of its Metabolite on Magnesium Ammonium Phosphate Crystals Fractal

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Chitinase producing microorganism was obtained from soil rich in chitin, and its metabolite was separated and purified by the method of salting out and chromatography respectively. Capillary electrophoresis was used to determine the homogenization of chitinase. The result showed that chitinase is homogenous to a certain extent, and its corresponding concentration is about 1.7145mg/ml. The method of double diffusion was employed to study the fractal of magnesium ammonium phosphate crystals (MAPs), and different concentration of chitinase solutions were added into reactive system of MAPs formation in order to investigate effect of chitinase on MAPs fractal. The results indicated that with the increasing of chitinase concentration, initial time of MAPs formation becomes longer and longer, crystal shape smaller and smaller, crystal mass less and less. It is suggested that chitinase could inhibit MAPs formation. MAP was characterized by XRD. The crystal is mainly magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4.6H_2O$) and a little magnesium ammonium phosphate hydrate ($MgNH_4PO_4.H_2O$).

Key words: Chitinase, MAPs, Fractal, Screening, XRD.

Chitinases are found in a wide range of organisms, such as animals, higher plants, and microorganisms. These enzymes catalyze the degradation of chitin polysaccharides, a linear β-1, 4-linked homopolymer of N acetylglucosamine, into many oligosaccharides with antibacterial activity[1-2] and have an amino terminal for binding chitin and a carboxyl terminal with catalytic activity[3-4]. In addition, chitinases can degrade cell wall of fungi into oligosaccharides with antibacterial activity. These oligosaccharides can inhibit pathogenetic microbe to grow[5-6]. Fänge *et al.* had discovered that chitinases have a tight association with digestion function of stomach, because these enzymes can degrade food "exoskeleton", such as polysaccharides, in stomach[7-8]. Lindsay[9] had thought that chitinases degrade some large repressor in intestine. So time when some substance for nucleation stays in vivo is shortened, and therefore, crystal growth, including inorganic crystal in urine stone, is inhibited.

Urinary stone disease is one of many diseases which make health and life quality of human beings weak. At present, mechanism of urinary stone formation has not been studied

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well[10]. Recently the study shows that urinary stone is made on epidemic cell of renal tubule under the action of organic matrixes, such as protein, polysaccharide, etc. In this process, organic matrixes provide many growth points distributed orderly to promote formation of sequential mineralization structure and linkage between minerals and parenchyma so as to nucleate themselves. Urinary stone is made up of inorganic crystals and organic matrixes, and inorganic crystal mainly includes calcium oxalate and MAP. At present, there have been a lot of researchers to make a correlative study on calcium oxalate crystal.

This work mainly carries out screening of chitinase producing strain and separating of its metabolite, and effect of different concentration of chitinase on MAPs fractal and the corresponding mechanism was also studied or discussed.

MATERIAL AND METHODS

Agents and equipments

DEAE, Chitin and NAG were acquired from sigma(USA). Foss AN300 and MPI-A capillary electrophoresis apparatuses were purchased from Foss (USA) and Ruimai (China) respectively.

Bacterium and Cultures

Bacillus N.Y. was isolated from the soil rich in chitin through the separated culture consisted of percent 0.5(W/V) casein (without vitamin), percent 0.2 chitin and percent 2.0 agar at pH 7.5, and fermented for chitinase production in shake culture composed of percent 2.5 soybean (W/V), percent 1.0 starch, percent 0.3 peptone, percent 0.2 yeast powder, percent 0.03 potassium dihydrogen phosphate, percent 0.01 magnesium sulfate and percent 0.15 calcium carbonate at pH 7.0.

Preparation of colloidal chitin

The method was referred to Nawani and Zhang Longxiang *et al*.[11-12].10g chitin powder was added in 180ml HCl (37%, W/V) at 25° under vigorous stirring for two hours. The suspension was poured into one liter percent 95 ice alcohol under vigorous stirring for 30min, and stored at below 20°. When used, 10ml of the suspension was centrifuged, and pellets were dissolved in 90ml 0.1mol/L sodium phosphate buffer (pH 6.0).

Purification of chitinase

Bacillus N.Y. was placed into 25ml shake culture in volume 250ml triangle flask and incubated at 28° 180r/min for 5 days. Then culture solution was centrifuged at 4000rev/min for 20min. The supernatant filtered by glass wool was crude chitinase. Crude extract fractionated with percent 50 saturation ammonium sulfate were centrifuged at 8000 rev/min for 20min. The pellets were dissolved in 0.8mol/l ammonium sulfate, and then the solution was centrifuged again at 15000 rev/min for 30min. The supernatant was collected and added into DEAE-fibin column(¢1.5cm*53cm) pre-equilibrated with pBS buffer. Firstly, the column was washed by PBS buffer, and then was eluted in grades by pBS buffer with 0.5mol/L sodium chloride. The portion with chitinase activity was pooled.

Determination of chitinase homogenization and concentration

Chitinase homogenization was determined by capillary electrophoresis at 10(T/S), and the corresponding sampling speed was magnified 3 folds. The concentration was identified by micro-Kjeldahl method with Foss AN300 apparatus. 2ml chitinase solution and 6ml concentrated H_2SO_4 was mixed and placed for 1.5 hours at 420 for sample digestion, and then the treated sample was added into Foss AN300 apparatus to determine the concentration of chitinase in the solution.

Enzyme activity assay

The assay was referred to Nawani method (Nawani *et al.* 2002). 0.5ml colloidal chitin reacted with 0.5ml chitinase solution at 50! for one hour. The volume of reducing sugar in the mixture was identified by DNS method (Zhang Longxiang et al. 1997). The enzyme volume was defined as one unit acquired for releasing 1 ìg NAG per min at 50°.

Formation of MAPs

In order to observe the effect of chritinase on MAPs fractal, double diffusion process in agar gel systems was used. The mixture of 2ml 1% agar and 2ml 0.6mol/l $NH_4H_2PO_4$ was added in the bottom of the tube. After it was frozen, 4ml 1% blank agar gel was added in the middle layer. Then the mixed solution was added in upper layer, which contained 2ml 0.6mol/l Mg^{2+} and different

concentration of chitinase, respectively. The chitinase prepared by above disposal were diluted as: 5, 4, 3, 2folds (Table 1).

RESULTS AND DISCUSSION

Screening strain and Determination

The well-done strain was screened by separated culture at 30° for 5-7 days with the largest ratio of strain to hydrolytic cycle (Fig.1). Through microscope (Enlarged by 16*40*8 folds) it looked like thin and long as *Bacillus*, and had some spores. Therefore, it is suggested it may be as a *Bacillus* (Fig.2), and is nominated *Bacillus* N.Y.

Determination of Chitinase

Chitinase homogenization was determined by MPI-4 capillary electrophoresis apparatus after ammonium sulfate salting out and DEAE-fibin column chromatography. The result was shown in Fig. 3 which had a distinct peak at approximate 315s. Whereas blank sample, fermentation solution without bacterium through the same disposal, had nothing at approximate 315 s. Therefore, chitinase solution was obviously homogeneous to a certain extent. 2ml chitinase digested by 6ml concentrated sulfuric acid for 1.5 hours at 420! was added into Foss AN300 apparatus to determine the concentration of protein in the solution. The result showed that volume of chitinase approximately equaled to 1.7145mg/ml.

Effect of Chitinase on Magnesium Ammonium Phosphate fractal

MAPs from tube E were characterized by XRD (Fig. 4). From Fig.4 MAPs were mainly made up of two type crystals, $MgNH_4PO_4.6H_2O$ and $MgNH_4PO_4.H_2O$. The blank arrows refer to $MgNH_4PO_4.6H_2O$, blue lines to $MgNH_4PO_4.H_2O$

Table 1. Composition of MAPs reactive system in different grades

Tube	Upper layer		Middle layer	bottom
A	150 µl fermentation culture without bacterium diluted by 2 folds.			2 ml 1% agar and 0.6mol $NH_4H_2PO_4$
B	150ìl Chitinase diluted by 5 folds	2 ml 0. 6mol/l Mg^{2+}	4ml 1% blank agar gel	
C	150ìl Chitinase diluted by 4 folds			
D	150ìl Chitinase diluted by 3 folds			
E	150ìl Chitinase diluted by 2 folds.			

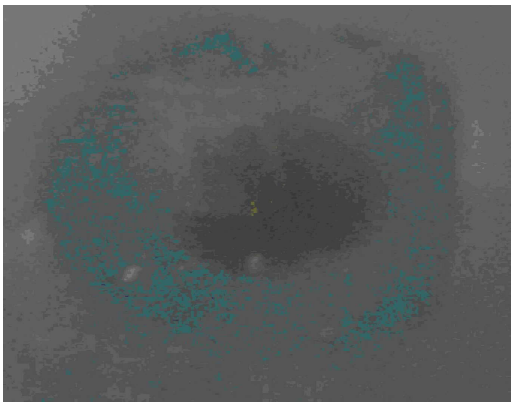


Fig. 1. Investigation of chitinase producing strain. The strain with the largest ratio of strain to hydrolytic cycle was screened by separated culture with chitin at 30 centigrade for 5~7 days.

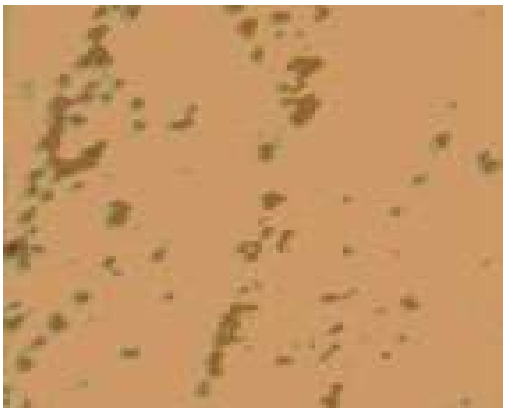


Fig. 2. Investigation of chitinase producing strain by microscope (Enlarged by 16*40*8). The screening strain in thin and long and has some spores

in Fig.4. It is suggested that $MgNH_4PO_4.6H_2O$ is the main composition of MAPs.

The fractal pattern of MAPs in tube A, B, C, D, E was shown in Fig 5a-5e. In tube A, point crystal appeared about 24h later, then its quantity increased and its shape changed to aciculate crystal. About 72h later, the shape and quantity of crystal stabilized (Fig.5a). In comparison with the crystal in tube A, the shape and quantity of crystal in tube B changed largely. It came out from the 70th hour and no change until 150th hour. The shape changed from point crystal

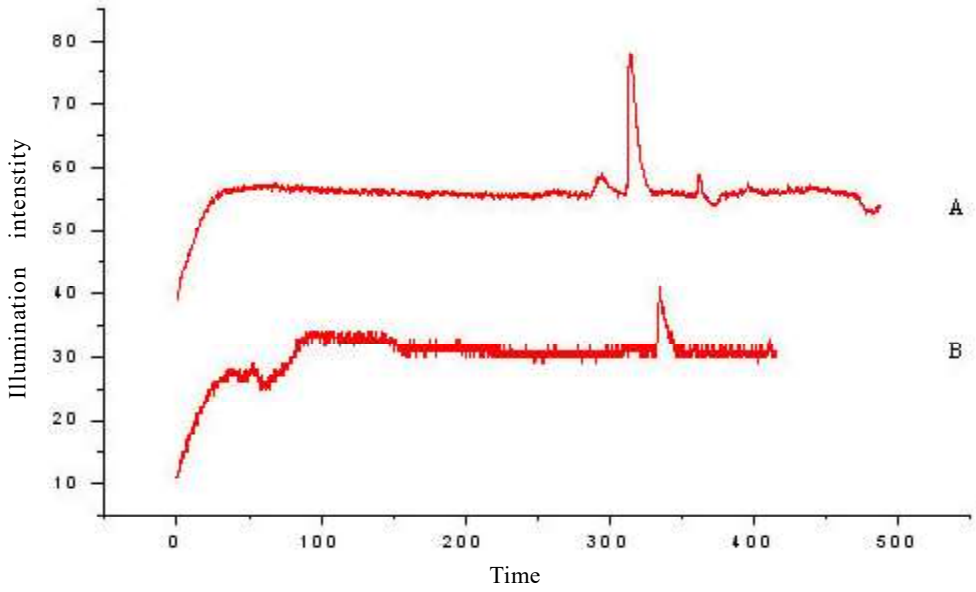


Fig. 3. Investigation of chitinase by capillary electrophoresis
A: sample of chitinase, B: Shake culture solution with inoculation as reference

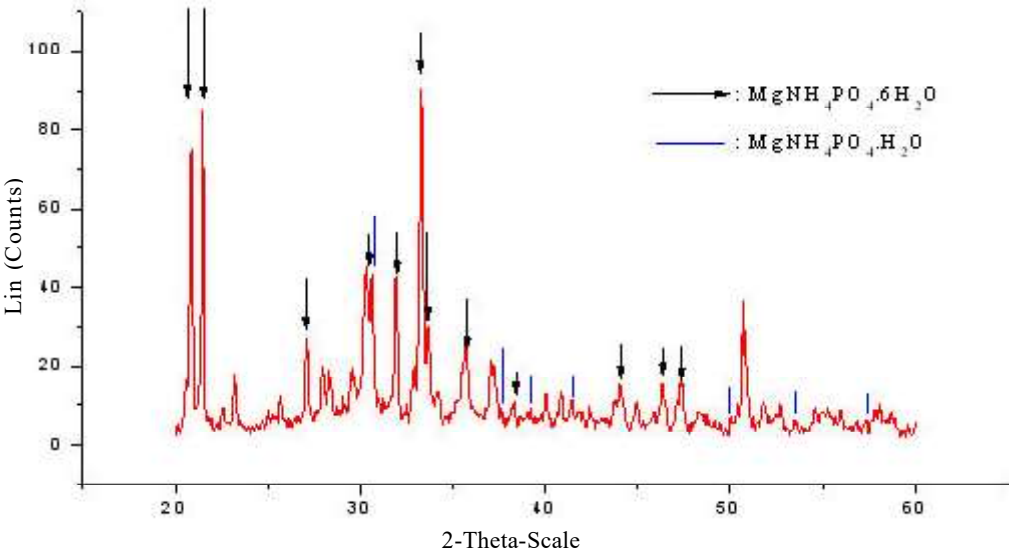
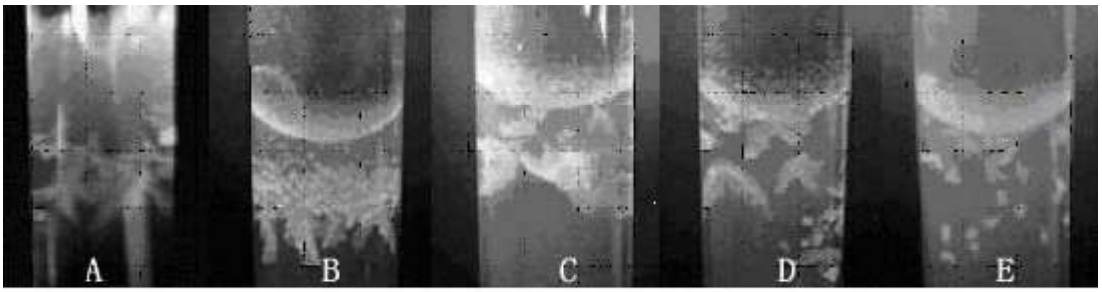


Fig. 4. XRD pattern of MAP crystals in double diffusion system

to quill-coverts crystal (Shown in Fig.5b). Obviously, the addition of chitinase made the fractal of MAPs change more. With the concentration of chitinase increasing, the time that crystal appeared became longer, the shape of crystal changed from aciculate to granular (Fig. 5c and 5e). The quantity of crystal decreased. The detected results were shown in Table 2.

Table 2. Effect of different concentration of chitinase on MAPs fractal

Tube	A	B	C	D	E
Initial time MAPs formation	24h	70h	80h	95h	122h
MAPs shape	Aciculate	Quill-coverts	Feathery	Feathery	Granular
MAPs mass/g	0.0213	0.0343	0.0173	0.0136	0.0100



A. MAPs looked like needles without chitniase
B. Chitinase was disluted by 5 folds. The number of MAPs was the most and their body small. MAPS were the most cowded like quill-coverts.
C. Citinase was dilluted by 4 flods. There were relatively little and loose MAPs with largest size like feathers.
D. Chitinase was diluted by 3 folds. MAPs were similar to B.
E. Chitinase was diluted by 2 folds. MAPs were the least and granular

Fig. 5. Effect of different concentration Chitinase on Magnesium ammonium phosphate fractal

From Fig.5a to Fig.5e, it was summarized that many different traits, for example:

1. It took more and more time for crystal to appear.
2. The shape of crystal changed more from Fig.5a to 5e.
3. The distributing trend of crystal is more and more disperse.
4. The mass of crystal is more and more decreased owing to above differences, it was suggested that chitinase may prohibit the crystal formation of MAPs. The reason may be that amino acid is the fundamental unit of chitinase, While amido, carboxyl are main composition in amino acid. So many groups of amido, carboxyl existed in chitinase, which could form hydrogen bond with Mg^{2+} to contest $H_2PO_4^-$. With the concentration of chitinase increasing, So the bonding force of $H_2PO_4^-$ with Mg^{2+} became weaker and weaker, which lead to the number of MAP decreased and the crystal became less and less. So we can make conclusion that chitinase could prohibit the fractal of MAPs.

CONCLUSION

Bacillus N.Y. was screened, and chitinase with the concentration of 1.7145mg/ml was obtained. Different concentration of chitinase has a significant effect on MAPs fractal. With the change of chitinase concentration initial time of MAPs formation, crystal shape and crystal mass changed largely. In a word, the fractal of MAPs could be inhibited by a certain concentration of chitinase.

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REFERENCES

1. Suginta W. Identification of chitin binding proteins and characterization of two chitinase isoforms from *Vibrio alginolyticus* 283. *Enzyme Microb Technol.* 2007; **41**:212-220.
2. Mayur Danny I. Gohel, Daisy K. Y. Shumb, Po Chor Tam. Electrophoretic separation and characterization of urinary glycosaminoglycans and their roles in urolithiasis. *Carbohydr. Res*. 2007; **342**: 79-86.
3. Yuichiro Kezuka, Manabu Ohishi, Yoshikane Itoh, et al. Structural Studies of a Two-domain Chitinase from *Streptomyces griseus* HUT6037 *J. Mol. Biol*., 2006; **358**: 472-484.
4. Lee, YS, Park IH, Yoo JS, *et al.* Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. *Bioresour Technol.* 2006; **98**: 2734-41.
5. Genfu Wu, Zhijian Yang. Isolation of bacteria producing chitin-degrading enzymes and preliminary studies on their optimum fermentative conditions. *J ZJU (Agric.& Life Sci.).* 2002); **28**: 641-645.
6. I.E. Cota , R. Troncoso-Rojas , R. Sotelo-Mundo, *et al*. Chitinase and β-1,3-glucanase enzymatic activities in response to infection by *Alternaria alternata* evaluated in two stages of development in different tomato fruit varieties. *Sci Hortic.* 2007; **112**: 42-50.
7. Fänge, R., Lundblad, G., Lind, K., *et al.* Chitinolytic enzymes in the digestive system of marine fishes. *Mar Biol.* 1979; **53**: 317-321.
8. Clark, J., Quayle, K.A., MacDonald, N.L., *et al*. Metabolism in marine flatfish. V. Chitinolytic activities in Dover sole, Solea solea. Comp. *Biochem Physiol* Pt B. 1988; **90:** 379-384.
9. Lindsay, G.J.H. Distribution and function of digestive tract chitinolytic enzymes in fish. *J Fish Biol*. 1984; **24**: 529-536.
10. George H N, Wenju Wu. Biomineralization mechanisms: a kinetics and interfacial energy approach. J Crys Grow. 2000; **211**: 137-142.
11. Nawani N N, Kapadnis B P , DAS A D, *et al.* Purification and characterization of a thermophilic and acidophilicchitinase from *Microbispora* sp. *J Appl Microbiol.* 2002; **93**:965-975.
12. Zhang Longxiang, Zhang Tingfang, Li Liangyuan. *Biochemistry Experimentation Technology*. Higher Education Press, Beijing 1997.