Antimicrobial Efficacy of Methanobactin against Bacillus Subtilis

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Abstract. Methanobactin is a novel extracellular fluorescent chromopeptide produced by methanotrophys. Its recent characterization has placed it in a new class of compounds, which is now known as chalkophores. They are analogous to iron-binding siderophores. The present study investigated the antimicrobial efficacy of copper-bound methanobactin (Mb-Cu) against Bacillus Subtilis in LB medium. Minimum inhibitory concentrations (MICs) determined that at 24 h for stationary-phase Bacillus Subtilis cultures in liquid LB medium (36℃) via use of a two-fold dilutions method, was 5 mg/mL. Minimum bactericidal concentrations (MBC) for Mb-Cu observed in the plate after 72 h at 36℃ was 10 mg/mL. Effect of different pH value (2 to 9) on antibacterial activity of Mb-Cu was measured by bacteriostatic circle method; the results showed that there was a non-liner relationship between the antimicrobial activity of Mb-Cu against Bacillus Subtilis and pH. Methanobactin was active in the range of pH 2～9 and has the best antimicrobial activity at near pH 6.0. Growth curve of Bacillus subtilis results showed that the maximum growth rate of Bacillus Subtilis was reduce and the lag phase was extended for 3 h.

Introduction

With the development of economy and society, people's living standards have been improving, because the food safety is more and more high. Food spoilage caused by microbial contamination has become the most important and general food safety issues, which not only decrease the nutritional value of food, but also cause food poisoning. Therefore, the prevention of contamination and growth of pathogenic foodborne microorganisms is a top priority assignment of economic and social development [1, 2]. As a kind of food additive, food preservative can inhibit microbial growth, avoid food spoilage within the warranty period and prolong the conservancy period of food. However, damage of chemical preservatives on human beings is beyond doubt. The food industry has paid the most attention to bacteriocins, such as nisin, polylysine, lactocin et al, it is apparent that other novel compounds similar to that of bacteriocins and siderophores may also possess antibacterial properties as well. One of these potential compounds is methanobactin. Methanobactin is a novel extracellular fluorescent chromopeptide, produced by the methane-oxidizing bacterium, Methylosinus trichosporium OB3b, an important organism involved in global carbon cycling and for single-cell protein production [3]. Under copper-limiting growth conditions, this compound accumulates to high amounts in the growth media. However, when copper is provided, copper-bound methanobactin (Mb-Cu) is rapidly internalized [4]. The structure of copper-bound methanobactin was N-2-isopropylester-(4-thionyl-5- hydroxyimidazolate)- Gly1-Ser2-Cys3-Tyr4-pyrrolidine-(4-hydroxy- 5-thionylimidazolate)-Ser5-Cys6-Met7 sequence with the empirical formula C45H12O14H62Cu (Fig. 1)[5].

The main goal of this paper is to learn whether copper-bound methanobactin has antibacterial effect on Bacillus Subtilis or not.

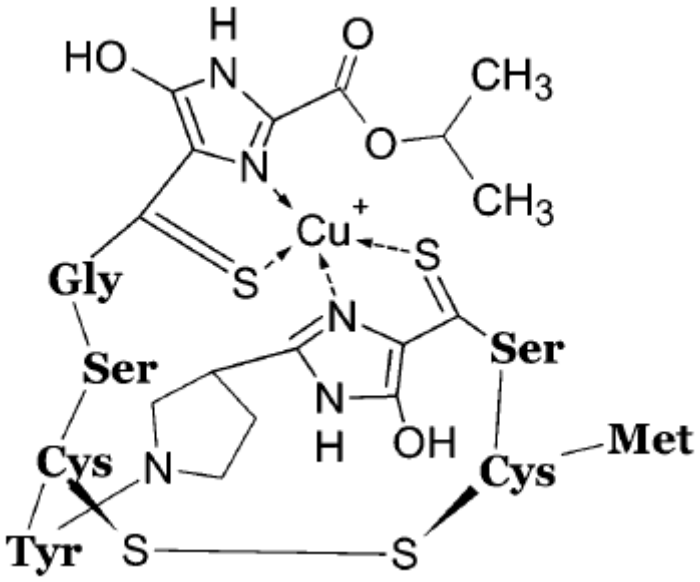

Fig. 1 Structure of Mb-Cu from *Methylosinus trichosporium* OB3b[5]

Materials and Methods

Microorganism and Medium. A methanotrophic strain named Methylosinus trichosporium IMV 3011 was obtained from the Institute of Microbiology and Virology (Kiev, Ukraine) [6]; Bacillus Subtilis, obtained from the key laboratory for food science and engineering, Harbin University of Commerce, was used throughout the experiment as an indicator.

Fermentation medium of test bacteria: copper-deficient nitrate minimal salts (NMS) medium [7].

Medium of indicator: LB medium, peptone 10.0 g, NaCl 10.0 g, yeast extract 5.0 g, distilled water 1000mL, solid medium containing 20g agar, adjusting the pH value of 7.0～7.2, sterilized 20 min at 121℃.

The Extraction and Purification of Methanobactin. Methylosinus trichosporium IMV 3011 were grown up in 3 L copper-deficient nitrate minimal salts (NMS) medium as previously described. A peristaltic pump fed the methanol as a source of carbon. After inoculation, researchers used a reaction temperature of 31 ℃, a stirring speed of 180 r/min and an oxygen flow of 500 mL/min at a constant methanol concentration of 0.1 % (V/V) by a methanol electrode. After 3 days of incubation, fermentation liquid of IMV 3011 was centrifuged at 8000 r/min for 15 min to pellet cells and take the supernatant. Afterwards, researchers added CuSO4 5H2O in the supernatant and then loaded onto a 4×30 cm Diaion HP-20 macroporous resin column. The column was then washed with deionized water for 30 min prior to elution with 60 % methanol [8]. Afterwards, the authors obtained the lyophilized copper-bound methanobactin samples (referred to as methanobactin throughout this paper) by freeze-dried immediately. Methanobactin samples were stored at ≤ -20 ℃ and held for no longer than 2 months. The concentration of Mb was measured by spectrophotometry after using Chrome Azure S[9].

Determination of the Minimum Inhibitory Concentrations (MICs) of Methanobactin. First, the Bacillus subtilis were inoculated in sterile liquid LB medium, table concentrator culture for 24 h (36 ℃, 180 rpm). Second, the inoculum was then diluted to achieve a concentration of 106 cfu/mL in tube. Subsequently, stock methanobactin final assay concentration of 40 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL by two-fold dilutions[10], Mb-Cu dilutions were transferred (each tube 1mL) to tube. Three hundred microliters of freshly cultured Bacillus subtilis culture was added to the each tube, in order to give a total volume of 5 mL. Negative controls used for sterility tests were non-inoculated liquid LB medium with or without Mb-Cu, while positive controls were inoculated liquid LB medium without Mb-Cu, 36 ℃ cultivation for the night. Afterward, in turn,

take out the 0.1 mL from no bacteria culture tube injection to sterile plate culture medium, 36 ℃ cultivation for 24 h. The MIC values for Mb-Cu were determined at plate where they were designated as the lowest Mb-Cu concentration at which no bacteria was observed in the plate. All inoculated plates were incubated at 36 ℃ for 72 h, the minimum bactericidal concentrations (MBC) for Mb-Cu were detemined at which no bacteria was observed in the plate.

Effect of pH Value on Antibacterial Activity of Methanobactin. *Bacillus subtilis* as indicator bacteria, and the concentration was 10^6 cfu/mL of it. By using HCl or NaOH, this paper adjusts the pH value of the methanobactin, ranging from 2.0 to 10.0, and determines the antibacterial activity of Mb-Cu by using inhibition zone method (punch to 2 cm in diameter). The measuring diameters on the three plates corresponding to a particular sample were averaged.

Effect of the Methanobactin on Growth curve of *Bacillus Subtilis.* Take out of freshly cultured *Bacillus subtilis*, culture was added to the methanobactin of liquid LB medium to achieve a concentration of 10^6 cfu/mL and added to the 1/2 MIC of Mb-Cu on it. Without methanobactin liquid, LB medium under the same conditions were used as a control. Growth rates and bacterial concentrations were determined by measuring optical density (OD) at 600 nm each 1 h (OD of 0.3 corresponds to a concentration of 10^8 cells per cm^3). With time as abscissa, OD_{600} value as ordinate, drawing the growth curve of *Bacillus subtilis.* Besides, data were analyzed by means of the software Matrix Laboratory 7.11.

Results

The minimum inhibitory concentrations (MICs) of Methanobactin. Table 1 showed that the minimum inhibitory concentration and the minimum bactericidal concentrations of methanobactin on the Bacillus subtilis. Indicator of Bacillus subtilis grew better in the plate at positive controls. After incubation for 24 h, at concentration of methanobactin 1.25 mg/mL to 2.5 mg/mL, the bacterial grew good in the plate, while, at 5 mg/mL to 40 mg/mL, the bacterial completely do not grew. After incubation for 72 h, no bacterium was observed in the plate which concentration 10 mg/mL to 40 mg/mL. Therefore, researchers can consider the minimum inhibitory concentration of methanobactin on the Bacillus subtilis was 5 mg/mL, and the minimum bactericidal concentrations was 10 mg/mL.

Table 1 The Experiment of Determining the Minimum Inhibitory Concentration of Methanobactin on the Bacillus Subtilis

Bacillus subtilis	Concentration of Methanobactin [mg/mL]						Positive Controls	Negative Controls
	40	20	10	5	2.5	1.25		
MIC	—	—	—	—	+	+	+	—
MBC	—	—	—	+	+	+		

Note: "+" express positive, colonies growth; "—" express negative, no colonies growth.

Effect on Antibacterial Activity of Methanobactin at Different pH Values. Comparative experiments were carried out to examine the effect of different pH value on antibacterial activity of methanobactin on the Bacillus subtilis. Fig. 2 showed that methanobactin has antibacterial effect on Bacillus subtilis at pH value range from 2 to 9. The most conducive pH for antimicrobial efficacy was pH 6, where the inhibition zone diameters were 29.12 mm. These results demonstrate the resistance acid and alkali of methanobactin.

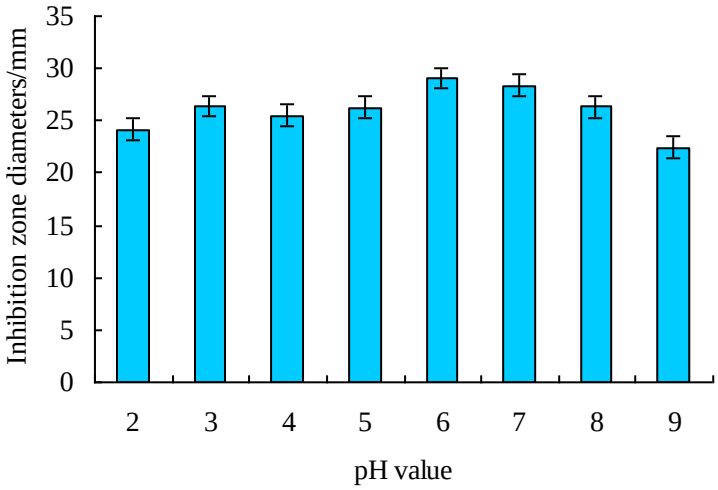

Fig. 2 Effect on Antibacterial Activity of Methanobactin at Different pH Values

Effect of the Methanobactin on Growth Curve of *Bacillus Subtilis.* Fig. 3 showed the growth curve of Bacillus subtilis and there are the maximum growth rate 0.2949 h-1 when at 5.3945 h, at the time the concentration of Bacillus subtilis corresponds to OD600 value of 0.6418, the lag phase was 3.2246 h. Compared with the results as shown in Fig. 4 and Fig. 3, the growth decreased, and the lag phase was extended for 3 h. These proved that 1/2 MIC methanobactin inhibits the growth of the Bacillus subtilis.

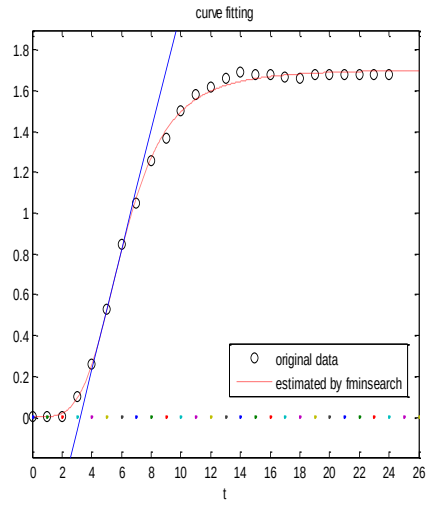

Fig. 3 Growth curve of Bacillus subtilis: there are the maximum growth rate 0.2949 h^{-1} when at 5.3945 h, the lag phase was 3.2246h

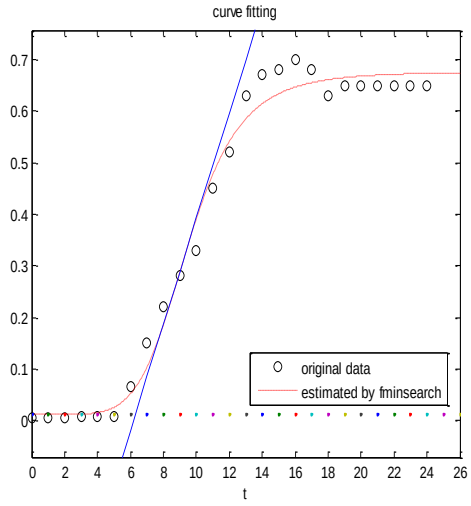

Fig. 4 Growth curve of Bacillus subtilis with addition of methanobactin: there are the maximum growth rate 0.1025 h^{-1} when at 8.9842 h, the lag phase was 6.2923h

Summary

The minimum inhibitory concentrations (MICs) of Bacillus Subtilis with methanobactin were 5 mg/mL and the minimum bactericidal concentrations (MBC) for Mb-Cu were determined to be 10 mg/mL. Mb-Cu against Bacillus Subtilis was active in the range of pH 2～9 and has the best antimicrobial activity at near pH 6.0. Growth curve of Bacillus subtilis with addition of methanobactin showed that the maximum growth rate of Bacillus Subtilis was reduced and the lag phase was extended

for 3 h. Therefore, methanobactin is a potential novel biopreservative for use against the Bacillus Subtilis.

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