# Effect of $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ on Growth and Sporulation of *Bacillus* sp. L15

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**Abstract.** The *Bacillus sp*. L15 isolated from a sea cucumber *Apostichopus japonicus* breeding pond could effectively reduce the contents of COD and $NH_4$-N in the pond water and significantly promoted the growth of *A.japonicus.* This study further tested the influence of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ on the reproduction and sporulation of vegetative cells in the *Bacillus* sp. L15 through an orthogonally designed experiment. The three ion types showed different impacts on the reproduction and sporulation of *Bacillus sp*. L15 cells. The $Ca^{2+}$ inhibited the reproduction of vegetative cells, but promoted spore maturation. The effect of $Mg^{2+}$ and $Mn^{2+}$ on vegetative cell reproduction and spore maturation depended on the range of ion concentration, and the optimal concentration for both ions promotion cell growth was 100 μg/L. This study suggests that the concentration of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ should be all at 100 μg/L in the basic freshwater fermentation medium for the culture of *Bacillus sp.* L15.

## Introduction

The *Bacillus sp*. L15 exhibiting maximum similarity (99%) with *Bacillus indicus* [20] at the 16S rRNA gene level was isolated from the sediment of sea cucumber (*Apostichopus japonicus*) ponds in Dalian, Liaoning Province, China. The *Bacillus sp.* L15 was identified as potential organics-degrading probiotics to reduce COD and $NH_3$-N levels in the pond sediment [20].The optimum seawater fermentation medium and culture conditions were screened by orthogonally designed experiments, and the amount of *Bacillus sp*. L15 could reach $10^{11}$ cfu/mL in the culture medium and a conventional culture condition [21] .

Considering seawater corrosion on the fermenter, the purpose of this study was to add some kind of ions in the freshwater fermentation medium to replace the seawater fermentation medium to culture *Bacillus sp.* L15. As there are many more types of cation in seawater than in freshwater, some of the cations may affect growth and sporulation of *Bacillus sp*. L15 cell. In this study, some cations are selectively added to the freshwater culture medium to enhance the growth and sporulation of vegetative cells.

Because the requirement of cations for growth and sporulation vary among bacterial species, this study tested the effects of ion type and quantity on the growth and sporulation of *Bacillus sp*. L15 in freshwater culture medium. According to the current literatures, $Ca^{2+}$ and $Mn^{2+}$ could induce sporulation and spore maturation of *B.subtilis* [4], *B.megaterium* [18] and *B. coagulans* var. thermoacidurans [1]. The $Mg^{2+}$ is essential for the formation of *Bacillus* spores [10,17]. In order to understand the role of some cations in spore-formation in the culture medium, this study (1) selected $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ as essential cations; (2)evaluated their impacts on vegetative cell growth and spore maturation of *Bacillus sp*. L15; and (3) determined their suitable adding dosage.

## Materials and methods

### Chemicals

In the present study, $CaCl_2$, $MgCl_2$ and $MnCl_2$, $FeSO_4•7H_2O$ were obtained fromTianjin Kemiou Chemical Reagent Co., Ltd. while other raw materials like soybean meal, wheat bran were purchased from Dalian Zhengtai feed Co., LTD. Peptone and yeast extract were all purchased from Beijing Star Biological Technology Co., LTD. All chemical reagents were of analytical reagent grade.

### Preparation of vegetative cell

The *Bacillus sp*. L15 was obtained from the Center for Technical Experiment of College of Fisheries and Life Science, Dalian Ocean University, Liaoning, China, maintained in a frozen culture at -80 ºC in 30% glycerol. The *Bacillus sp.* L15 from the -80℃ stock was grown for 24 h in the Zobell Marine Agar (ZMA) agar slant culture-medium containing 5g/L peptone, 1g/L yeast extract and 0.01g/L $FePO_4•7H_2O$ in 1L pre-precipitated seawater at pH 7.0-7.5 (adjusted with NaOH) at 25℃.This initial culture was used to inoculate 150 mL ZMA medium in a 250 mL flask shaken for 12h at25℃. Ten ml second generation culture broth was transferred to a fresh 150 ml ZMA medium and cultured again for 12h before use.

### Growth curve and spore mature rate of *Bacillus sp*. L15

According to 1% (v/v) inoculation proportion, the broth with culture of *Bacillus sp*. L15 for 12h was transferred to a 250 ml flask containing 120 ml of basic fermentation medium (BFM: soybean meal 2.5 g, wheat bran 2.5 g, distilled water 1L, pH 7.0 - 7.5) as the treatment group. There were no bacteria added in another BFM as the control group. Both groups were in triplicate and incubated in a shaker at 25℃ and 150 rpm.

In the first 24h during the period of culture, every 2 h, 5 ml of the broth was collected and settled for 20 min, and then the absorbance (OD) of supernatant was measured with a 721 spectrophotometer (λ=600nm, 1cm glass cells). Meanwhile, 10μl of shaken broth was added onto a glass slide, and then stained with the malachite green stain-method (Dong 2001). The numbers of vegetative cells and spores were counted for 10 fields on the glass slide under a microscope (100, Olympus, AB-2) to calculate the rates of spore maturation. After 24h, the process was repeated once every 12 h for 120 h. The difference of absorbance OD between the treatment and control group reflected the growth of Bacillus sp. L15. The rates of spore maturation (R) were calculated as follows:

$$R=N/（N+M）\times 100\% \quad (1)$$

Where, N is the number of mature spore, and M is the number of vegetative cells.

### Effects of cations

An orthogonal $L_9(3)^4$ test design was used to investigate the optimal dosages of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$. As seen from Table 1, the experiments were carried out with 3 levels of each kind of cation. Salts were added to 120 ml BFM in 250 ml flasks, and then the media were sterilized at 121℃ for 20 min. Each group experiment included two treatments and one control group. The experimental protocols in the treatment group and the control group test methods, including Bacillus sp. L15 inoculation, OD determination of bacterial growth, calculations of spore mature rates, were as the same as the method above section. The absorbance (OD) of supernatant and rates of spore maturation were tested after cultivation for 48 h, 72 h, 96 h and 120 h.

### Statistical analysis

OD of bacterial growth and rates of spore maturation were presented as mean ± SE (standard error). The data were statistically analyzed by SPSS 19.0, and the differences of bacterial growth and rates of

spore maturation among different levels of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ were analyzed by Duncan multiple comparison. The significance level was set at $P = 0.05$

## Results

### Growth curve of *Bacillus* sp. L15

The growth curve of Bacillus sp. L15 in BFM is shown in Fig.1. The lag phase duration was approximately 4 h. The exponential phase duration was approximately 22 h, ranging from 4 to 36h post inoculation. The stationary phase duration was72 h from 36 to 108 h and the aging phase started at 108h post inoculation.

### Sporulation time and spore mature rate of *Bacillus* sp. L15

In BFM, Bacillus sp. L15 could form spores normally, but the spore formation and mature rates were lower (Fig.2). The *Bacillus* sp. L15 began to form spores at the late exponential phase (28 h) and a small part of spores matured at 72h. The sum of spore formation and mature rates was 23.7% at 72 h, 31.39% at 96 h and 41.91% at 120 h.

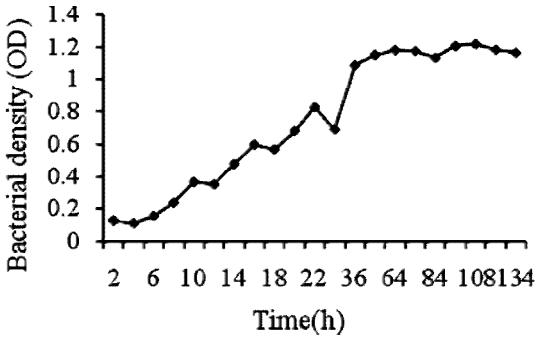


Fig.1 Growth curve of L15 strain in 2216E medium

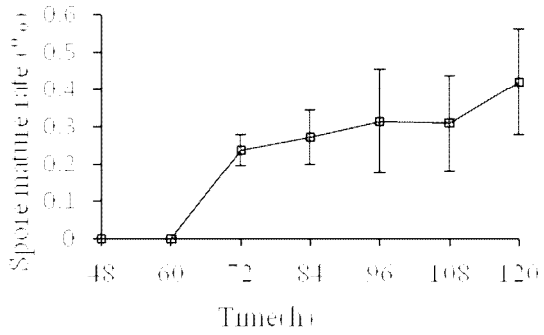


Fig.2 Spore rates of L15 strain in 2216Emedium

### Effects of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ on the cell growth of *Bacillus* sp. L15

Results and analysis of orthogonal $L_9(3)^4$ test are shown in Table 1. The cell growth of *Bacillus* sp. L15 decreased with the increasing of $Ca^{2+}$ dosage, but was promoted by lower dosage $Mn^{2+}$ and inhibited by higher dosage $Mn^{2+}$. After 72 h, the effects of $Mg^{2+}$ on the growth of *Bacillus* sp. L15 was similar with $Mn^{2+}$. The cell growth of *Bacillus* sp. L15 showed no significant difference among different concentrations of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ ($p>0.05$).

In conclusion, to obtain a higher number of Bacillus sp. L15, the optimum concentration of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ was 0 μg/L, 100 μg/L and 100 μg/L, respectively.

The data are presented as mean ± SE. The significant differences between each level of the same ion are indicated on the lower case letters ( $P < 0.05$), The follow table was the same.

**Table1** Effects of different dosages of $Ca^{2+}$, $Mn^{2+}$, $Mg^{2+}$ on *Bacillus sp*. L15 growth(OD)

| | | Bacterial growth OD (Mean ± SE) | | | |
|---|---|---|---|---|---|
| Factors | Levels | 24h | 48h | 72h | 96h |
| $Ca^{2+}$ | 1 | $0.352 \pm 0.137^a$ | $1.083 \pm 0.232^a$ | $1.376 \pm 0.070^a$ | $1.462 \pm 0.077^a$ |
| | 2 | $0.120 \pm 0.198^a$ | $0.471 \pm 0.620^a$ | $1.157 \pm 0.178^a$ | $1.351 \pm 0.040^a$ |
| | 3 | $0.037 \pm 0.157^a$ | $0.469 \pm 0.539^a$ | $0.959 \pm 0.362^a$ | $1.309 \pm 0.166^a$ |
| $Mn^{2+}$ | 1 | $0.112 \pm 0.305^a$ | $0.353 \pm 0.666^a$ | $1.012 \pm 0.422^a$ | $1.287 \pm 0.143^a$ |
| | 2 | $0.315 \pm 0.104^a$ | $1.140 \pm 0.104^a$ | $1.317 \pm 0.152^a$ | $1.453 \pm 0.074^a$ |
| | 3 | $0.083 \pm 0.100^a$ | $0.530 \pm 0.100^a$ | $1.163 \pm 0.164^a$ | $1.382 \pm 0.084^a$ |
| | 1 | $0.226 \pm 0.220^a$ | $0.739 \pm 0.506^a$ | $1.167 \pm 0.184^a$ | 1.376 ± 0.066a |

| | | | | | |
|---|---|---|---|---|---|
| $Mg^{2+}$ | 2 | 0.139 ± 0.225[a] | 0.650 ± 0.619[a] | 1.257± 0.171[a] | 1.420 ±0.098a |
| | 3 | 0.144 ± 0.236[a] | 0.634 ± 0.676[a] | 1.068 ± 0.456[a] | 1.327 ± 0.098a |

The data are presented as mean ± SE. The significant differences between each level of the same ion are indicated on the lower case letters ( $P < 0.05$), The follow table was the same

**Effects of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ on spore maturation**

Results and analysis of orthogonal $L_9(3)^4$ test are shown in Table 2. In the ninth experiments, the highest mature rate of spore was up to 70.8% at 72 h and 95.7% at 96 h, which indicated that $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ were benefit to spore maturation.

However, the effects of $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$on spore maturation of *Bacillus* sp. L15 were different. The effects of $Mn^{2+}$ and $Mg^{2+}$on spore maturation were similar with their effects on the growth of *Bacillus* sp. L15, and the mature rates of spore were the highest in 100 μg/L. The effects of $Ca^{2+}$on spore maturation were different with the effect of it on the growth of *Bacillus* sp. L15. $Ca^{2+}$ was beneficial to spore maturation of *Bacillus* sp. L15. The mature rates of *Bacillus* sp. L15 spore showed no significant difference among different concentrations of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ ( $p > 0.05$).

**Table2** Effects of different dosages of $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ on *Bacillus sp*. L15 spore mature rates

| | | Spore mature rates (Mean ± SE) | |
|---|---|---|---|
| Factors | Levels | 72h | 96h |
| $Ca^{2+}$ | 1 | 0.067 ± 0.115 | 0.692 ± 0.220 |
| | 2 | 0.286 ± 0.261 | 0.625 ± 0.500 |
| | 3 | 0.396 ± 0.271 | 0.859 ± 0.015 |
| $Mn^{2+}$ | 1 | 0.473 ± 0.256 | 0.804 ± 0.104 |
| | 2 | 0.204 ± 0.181 | 0.9058 ± 0.058 |
| | 3 | 0.072 ± 0.125 | 0.466 ± 0.411 |
| $Mg^{2+}$ | 1 | 0.155 ± 0.138 | 0.526 ± 0.420 |
| | 2 | 0.242 ± 0.256 | 0.885 ± 0.026 |
| | 3 | 0.352 ± 0.354 | 0.764 ± 0.255 |

In conclusion, to obtain the highest spore mature rate of *Bacillus* sp. L15, the optimum concentration of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ was 500 μg/l, 100 μg/l and 100 μg/l, respectively. Conclusion: considering the overall effects of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ on the cell growth and spore maturation, the present study showed that the concentrations of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$should be about 100 μg/L each. On the basis of this study, it was necessary to conduct further experiments to determine the optimum dosage.

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**Disscussion**

**Growth curve and spore mature rates of *Bacillus* sp. L15**

In a batch pure culture, the growth curve of bacteria could be used to understand the starting time and physiological characteristics at different periods of bacterial growth, including the lag phase duration, exponential growth rate, stationary growing time, as well as the morphological and physiological characteristics, metabolic product characteristics during each period. Bacterial growth curve differs between bacterial species, culture conditions [7], inoculation quantities [6], and medium types [3]. Therefore, the growth curve of *Bacillus* sp. L15 was determined under new formulation of culture medium and conditions. The lag phase duration, exponential growth rate, stationary starting time, and spore production and mature time were determined from the growth curve. Based on the change of growth curve, the effects of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ on vegetative cell growth and spore maturation at each sampling time were determined.

It was visible that the duration of lag phase of *Bacillus* sp. L15 was longer in the freshwater BFM than in ZMA and this is due to the to the difference in ionic composition of the freshwater BFM and ZMA [2].The spore formation began in the late exponential phase, but the mature time of spores began in the aging period, suggesting that the sampling time should occur at a later growing stage. The mature

rates of spore were lower (<50% in 120 h), which could be due to a lack of some minerals in the freshwater culture medium during the sporulation of *Bacillus* sp. L15.

**Influence of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ on cell growth and spore maturation of *Bacillus* sp. L15**

Although cations can affect cell growth and spore maturation of some bacteria, the requirement of cation species and quantity varies among bacterial species. $Ca^{2+}$ is a bacterial intracellular ubiquitous mineral ion [8]. Therefore, when the culture medium is $Ca^{2+}$ deficient, the addition of $Ca^{2+}$ would be theoretically beneficial to bacterial growth. However, the effects of $Ca^{2+}$ on bacterial growth were different. Mah et al. [8] and Qian et al. [12] reported that addition $Ca^{2+}$ to the culture medium could promote bacterial growth, but Sun et al. [16] reported that $Ca^{2+}$ inhibited bacterial growth. The discrepancy between these study results may also be related to bacterial species, medium composition and $Ca^{2+}$ dosage.

$Ca^{2+}$ is an important component of bacillus spores, which is closely related to the heat resistance of spores [9,10,11] and sporulation and spore maturation [8,16,19]. The experiment result indicated that adding $Ca^{2+}$ in the BFM inhibited *Bacillus* sp. L15 growth, but promoted sporulation and spore maturation. Therefore, it was imperative to add $Ca^{2+}$ in the BFM to culture *Bacillus* sp. L15.

The effects of $Mg^{2+}$ on the vegetative cells growth and spore maturation of *Bacillus* sp. L15 were similar to $Ca^{2+}$, which was also related to bacterial species and $Mg^{2+}$ compound types and dosage. Song et al. [14] reported that $MgSO_4$ could promote *Pseudomonas fluorescens* growth, but in 2006 the author reported that $MgCl_2$ did not have any effect to this bacteria growth. Difference between both of study results may also be due to the fact that $SO_4$-$^2$ increased the growth of planktonic cells of *Pseudomonas fluorescens* [14]. Therefore, when we test whether one cation has influence on bacterial growth, we should choose the cationic compound in which the anion does not effect on bacterial growth. In contrast to Song's reports, Sun et al. [16] reported that adding 0.3% $MgSO_4.7H_2O$ in the medium could inhibit *B.natt* growth, but Yao et al. [19] reported that adding less than 0.03% $MgCl_2$ in the medium could promote the growth of *B.subtilis* BS-MM03 vegetative cells.

Mah et al. [8] reported that $Mg^{2+}$ was the basic ion for sporulation of the *Bacillus* genus, but some conclusions are opposite. Sun et al. [16] reported that adding 0.3% $MgSO_4.7H_2O$ in the medium inhibited *B.natto* sporulation, and the rate of sporulation was 15% lower than in the medium without $MgSO_4.7H_2O$. Yao et al. [19] and Mah et al. [8] reported that $MgCl_2$ could promote bacterial sporulation. This paper indicates that adding 0.5% $MgCL_2$ in BFM could promote *Bacillus* sp. L15 vegetative cell growth and spore maturation, but more than 0.5% concentration would inhibit vegetative cell growth and spore maturation of *Bacillus* sp. L15.

Although $Mn^{2+}$ is accessory factor of many enzymes in bacterial cells, such as superoxide dismutase and L-arabinose isomerase [13], bacterial growth and sporulation do not need the involvement of all these enzymes. The effects of $Mn^{2+}$ on the vegetative cell growth and spore maturation of bacteria were also related to bacterial species and $Mn^{2+}$ compound types and dosage. Sun et al. [16] reported that $MnSO_4$•$H^2O$ could promote *B.natto* vegetative cell growth and sporulation, and the number of vegetative cells and rates of sporulation increased with the increase of $Mn^{2+}$ concentration. Bacterial number reached the highest in the medium containing 1.2 ~ 2.4 mmol $Mn^{2+}$/L (i.e., 0.2 ~ 0.4 g $MnSO_4$•$H_2O$ /L) and the rate of spore maturation achieved more than 98%. However, when the $Mn^{2+}$concentration exceeded 2.4 mmol/L, sporulation was inhibited. Song et al. [15] reported the same result for *B.subtilis* in the range of 0.0～3.6 mmol/L $MnSO_4$• $H_2O$. Yao et al. [19] reported that *B.subtilis* BS - MM03 vegetative cells and the spore number also increased in the range of 0.001 - 0.015% $MnCl_2$. Amaha et al. [1], Charney et al.[4] and Weinberg [18] reported that both of $MnSO_4$ and $MnCl_2$ could promote most *Bacillus* growth and spore maturation. But Mah et al. [8] reported no effect of $MnCl_2$on *Clostridium sporogenes* sporulation.

This study confirmed that adding certain concentration of $MnCl_2$ to BFM could promote *Bacillus* sp. L15 growth and sporulation.

**Conclusion**

it was feasible that using wheat bran and soybean meal freshwater medium cultured ***Bacillus*** sp. L15, but some mineral salts must be added, otherwise the number of bacteria and spore mature rate were lower. The experiments showed that adding certain concentration of $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ in the BFM could enhance cell quantity and rate of spore maturation of ***Bacillus*** sp. L15. This result contribute to the understanding on the requirement of cation ions in the culture of bacteria that could reduce COD and ammonia nitrogen in pond water.

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