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Studies on Solubility and Stability of Oligochitosan in Effective Microorganisms

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Abstract [**Objective**] This study aimed to explore the synergistic effect of oligochitosan and EM bacteria and provide a theoretical basis for its application in animal health, aquaculture, sewage treatment and other fields. [**Method**] Different concentrations of oligochitosan were added into EM bacteria liquid and the stability was preliminarily studied. [**Result**] Addition of oligochitosan with appropriate proportion had played a supporting role in the stable storage of EM bacteria liquid and had shown certain inhibition effect on flatulence of EM bacteria liquid. Addition of oligochitosan had no significant effect on the quality and re-fermentation of EM bacteria. [**Conclusion**] The study indicated that oligochitosan could be utilized in conjunction with EM bacteria liquid, having a promising practical potential application.
Key words Oligochitosan; Effective microorganisms (EM); Stability

EM (Effective Microorganism) is a new type of composite microbial active inoculants developed by a Japanese professor in University of the Ryukyu in the 1980s, which was composed of 80 microorganism species in 10 genuses of five major flora including photosynthetic bacteria, acetobacter, actinomyces, lactic acid bacteria and yeast. These microorganisms had formed an efficient microbial flora with complex composition, stable structure, extensive functions and no toxic side effect through their synergistic effect and proliferation relationship. Currently, EM products were widely used in agriculture, animal husbandry, aquaculture, environmental cleanup and other fields[1–2].

Chitosan is a natural polymer produced via deacetylation of chitin. In comparison with chitosan, oligochitosan has a relatively low molecular weight, higher water solubility and biological activity. In the field of life sciences, a large number of previous research papers have indicated that chitosan, especially oligochitosan, has functions such as regulating lipid metabolism in animals and improving nutrition and metabolic efficiency of animals[3]. In recent years, researchers have found that the addition of chitosan in animal feeds had positive effect[4–5]. Chitosan has been used as antiseptic in production of soybean paste, soy sauce, Japanese –style noodles and Chinese noodles and fermentation–control reagents in production of pickled products. In Japan, the government has approved the direct addition of chitosan in food and medicine, food and feeds containing chitosan have been widely produced in Japan, and the technology to prolong the shelf life of food by using chitosan has been patented. In Canada, carboxymethyl chitosan has been approved to be used as food antiseptic in coating preservation of eggs and fruits (such as pears and apples). Commercialized antifungal coating materials with chitosan as matrix have been sold as preservative for fresh fruits[6].

Allan *et al*.[7] had first proposed the broad-spectrum antibacterial activity of chitosan in 1979. Since then, many scholars conducted extensive and in-depth studies on the antibacterial activity of chitosan and found that chitosan has antibacterial activity against various bacteria, yeast and fungi [8]. Addition of 0.5% or 1.0% of chitosan in feeds for Allogynogenetic silver crucian carp could increase the lysozyme activity (P<0.01) and leukocyte phagocytosis activity (P<0.01)[9]. Different additive amounts of oligochitosan have various effects on feed metabolic properties of animals. Previous experiments showed that adding 0.5%–1.0% of oligochitosan could improve the utilization efficiency of feeds by a factor of more than 2.0%[3].

In this study, different concentrations of oligochitosan were added into EM bacteria liquid to study the stability of EM bacteria liquid preliminarily, explore the synergistic effect of oligochitosan and EM bacteria and provide a theoretical basis for its application in animal health, aquaculture, sewage treatment and other fields.

Materials and Methods

Materials

EM bacteria liquid was introduced from Japan and specifically cultured in Jiaxing Korui Biotechnology CO., LTD. Specific medium for determination of total number of viable cells contained 1% of glucose, 1% of peptone, 0.5% of soluble starch, 0.1% of KH_2PO_4, 0.05% of $MgSO_4 \cdot 7H_2O$, 0.001% of $FeSO_4 \cdot 7H_2O$, 0.001% of $CaCl_2 \cdot 6H_2O$ and 2% of agar; EM production medium contained 1% of glucose, 0.5% of peptone, 0.5% of soluble starch, 0.1% of KH_2PO_4, 0.05% of $MgSO_4 \cdot 7H_2O$, 0.001% of $FeSO_4 \cdot 7H_2O$ and 0.001% of $CaCl_2 \cdot 6H_2O$. Oligochitosan microp-

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owder was offered by Zhejiang Zhongke Radiation Polymer Materials Research and Development Center, with an average particle size of about 20 μm, deacetylation degree of 85% and average molecular weight less than 50 000, which was mainly used as feed additives.

Apparatus and equipments

Ordinary pH meter, disinfection and sterilization pot, clean bench and biological microscope were used in this experiment.

Methods

Effect of oligochitosan powder on EM bacteria liquid The pH in EM bacteria liquid after adding different amounts of oligochitosan was measured, dissolution of oligochitosan and variation of flora were also observed. It was found that oligochitosan powder was dissolved directly in EM bacteria liquid with contents of 0.1%, 0.25%, 0.5% and 1.0%, respectively. Mixture solution was shaken for 18 h to be fully dissolved and kept under seal at a constant temperature of 34 ℃. The total number of viable cells and pH were determined by using coated plate method after 1 and 3 weeks, respectively.

Effect of acidic oligochitosan solution on EM bacteria liquid Oligochitosan powder was pre-dissolved in different acidic solutions[10]: ① 6% acetic acid solution (10% of oligochitosan was dissolved, pH=4.0); ② 5% lactic acid solution (10% of oligochitosan was dissolved, pH=3.3). Additive contents of oligochitosan in EM bacteria liquid were 0.1%, 0.25%, 0.5% and 1.0%, respectively. Oligochitosan powder was fully mixed with EM bacteria liquid and kept under seal at a constant temperature of 34 ℃. Variation of pH was observed after a week and the total number of viable cells was determined by using the coated plate method. In addition, the total number of viable cells in incompletely sealed EM bacteria liquid, which was cultured under normal circumstances, was determined by using the coated plate method after 1 week, 3 weeks and 1 month, respectively.

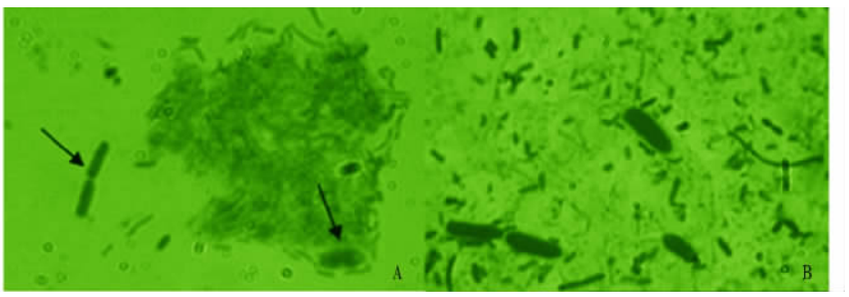
Re-fermentation experiment of EM bacteria liquid with the addition of oligochitosan EM bacteria liquid contained directly dissolved oligochitosan powder and EM bacteria liquid mixed with acid solutions of oligochitosan were used for fermentation experiment. 5 ml of experimental liquid was inoculated in 250 ml of medium for fermentation culture, and the total number of viable cells was determined by using coated plate method after 1 week. Flatulence of EM bacteria liquid with the addition of oligochitosan was observed and microscopic examination was conducted to observe the variation of microbial morphology.

Results and Analysis

Variation of pH and flora in EM bacteria liquid with direct dissolution of oligochitosan powder

It was found that the morphology of yeast varied in EM bacteria liquid with direct dissolution of oligochitosan powder (Fig.1). Compared with EM bacteria liquid without the addition of oligochitosan, EM bacteria liquid with direct dissolution of oligochitosan powder basically contained the same flora, but the yeast was decomposed with decreasing number, and the volume of bacteria liquid was reduced.

As can be seen from Fig.3, the number of viable cells in each experimental sample was all reduced after experiments compared with before. However, the number of viable cells in EM bacteria liquid containing 0.1%, 0.25% and 0.5% of oligochitosan was respectively 14.7%, 15.9% and 37.6% more than that in control group after a week; the number of viable cells in EM bacteria liquid containing 0.1%, 0.25% and 0.5% of oligochitosan was respectively 41.9%, 448.0% and 267.0%



A, EM bacteria liquid with addition of oligochitosan; B, EM bacteria liquid without addition of oligochitosan.

Fig.1 Variation of flora in EM bacteria liquid at 1600 × magnification

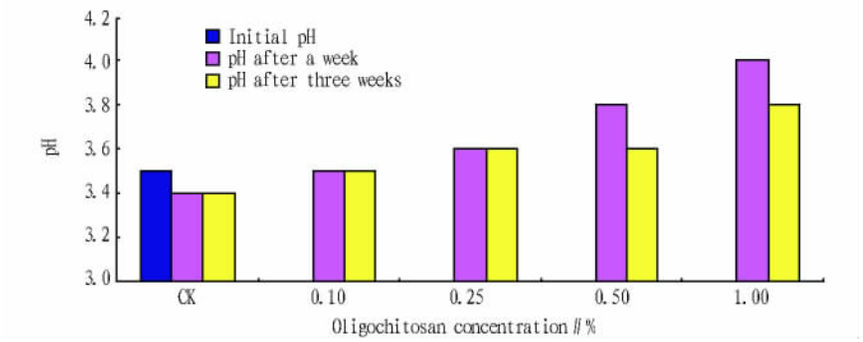


Fig.2 Variation of pH in EM bacteria liquid containing different concentrations of oligochitosan

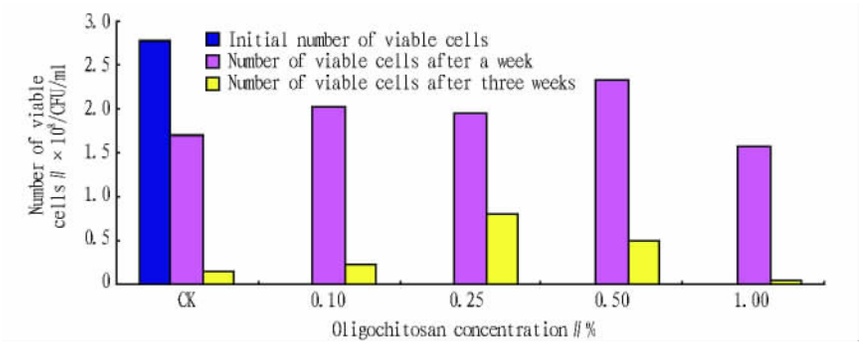


Fig.3 Variation of number of viable cells in EM bacteria liquid containing different concentrations of oligochitosan

more than that in control group after three weeks.

Decrease rate of the number of viable cells in EM bacteria liquid containing 0.1% , 0.25% and 0.5% of oligochitosan was smaller than that in control group, while the reduced number of viable cells in EM bacteria liquid containing 1.0% of oligochitosan was the maximum. After three weeks, the number of viable cells in EM bacteria liquid varied largely. The number of viable cells in EM bacteria liquid containing 0.25% and 0.5% of oligochitosan was several times of that in control group, while the number of viable cells in EM bacteria liquid containing 1.0% of oligochitosan was the minimum, which showed that there wasn't a linear relationship between the concentration of oligochitosan and the number of viable cells. The higher the concentration of oligochitosan was, the higher the pH in EM bacteria liquid would be (Fig.2), which might be resulting in the faster reduction of the number of viable cells in EM bacteria liquid containing 1.0% of oligochitosan. The other reason caused the reduction of the number of viable cells in EM bacteria liquid with addition of oligochitosan might be the flocculation of chitosan, which caused some viable cells in EM bacteria liquid sinking to the bottom of the container.

Variation of pH and flora in EM bacteria liquid mixed with different acid solutions of oligochitosan

As can be seen from Fig.4, lactic acid solution of oligochitosan had the minimum effect on the pH of EM bacteria liquid; while pH of EM bacteria liquid mixed with acetic acid solution of oligochitosan had increased with the increasing concentration of oligochitosan.

The number of viable cells in EM bacteria liquid mixed with 0.1% and 0.5% of acetic acid solution of oligochitosan was18.7% and 31.2% more than that in control group, respectively; the number of viable cells in EM bacteria liquid mixed with 0.1% , 0.25% , 0.5% and 1.0% of lactic acid solution of oligochitosan was 94.4% , 61.1% , 100.0% and 16.6% more than that in control group, respectively(Fig.5). Addition of 0.1%, 0.25% and 0.5% of lactic acid solution of oligochitosan had substantially increased the amount of lactic acid bacteria in EM bacteria liquid while greatly declined the amount of yeast, which might be due to the promotion of lactic acid solution on the re-growth of lactic acid bacteria in EM bacteria liquid; addition of acetic acid solution of oligochitosan had substantially declined the amount of yeast but slightly affected the lactic acid bacteria, and the preservation effect was relatively better with 0.1% and 0.25% of acetic acid solution than with 0.5% and 1.0% of acetic acid solution.

As can be seen from Fig.6, experimental sample without addition of oligochitosan was packaged and stored at room temperature for a month, the number of viable cells in EM bacteria liquid had decreased by 50% , which had shown significant postponement compared with the reduction of the number of viable cells in control group under experimental conditions, indicating that the reduction of the number of viable cells of the same sample varied at different temperatures. At room temperature, incompletely sealed products were better stored. In addition, the results also revealed that shelf life of EM bacteria was relatively shorter than the solid microbial feed additives, so that it should be used as soon as possible to ensure the best results.

Re-fermentation experiment of EM bacteria liquid with addition of oligochitosan

EM bacteria liquid with addition of oligochitosan powder was stored for 2

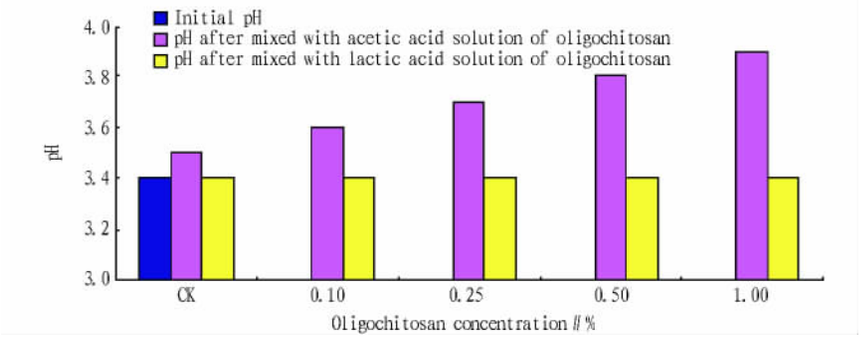


Fig.4 Variation of pH in EM bacteria liquid mixed with different acid solutions of oligochitosan

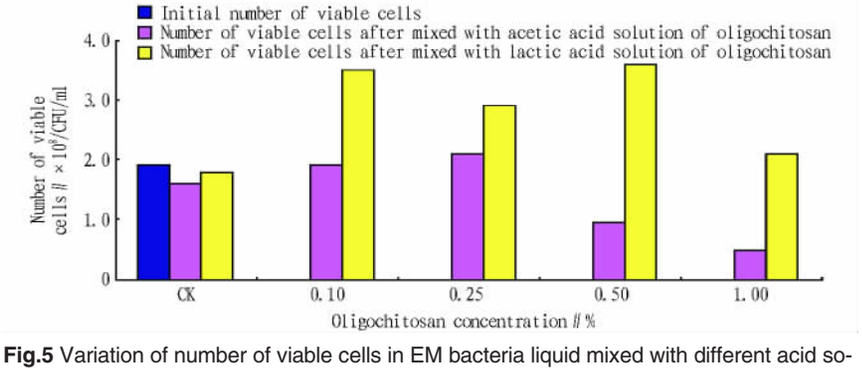


Fig.5 Variation of number of viable cells in EM bacteria liquid mixed with different acid solutions of oligochitosan

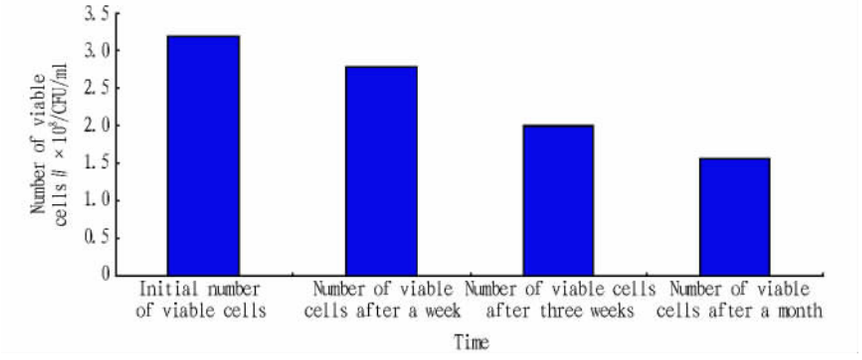


Fig.6 Variation of number of viable cells in EM bacteria liquid without addition of oligochitosan

Table 1 Number of viable cells in re-fermented EM bacteria liquid with addition of oligochitosan

Oligochitosan concentration // %	Yeast // CFU/ml	Lactic acid bacteria // CFU/ml	pH
0.1	9×10^6	3.0×10^8	4.4
0.25	4×10^6	3.7×10^8	4.2
0.5	$<1\times10^6$	6.3×10^8	4.0
1.0	$<1\times10^6$	5.4×10^8	3.6

All values are means of three replicates.

Table 2 Number of viable cells in re-fermented EM bacteria liquid mixed with 0.5% of acid solutions of oligochitosan

Treatment	Yeast // CFU/ml	Lactic acid bacteria // CFU/ml	pH
Control group	1.1×10^7	2.3×10^8	4.4
Lactic acid group	1.2×10^7	1.1×10^8	4.3
Acetic acid group	2.2×10^7	1.5×10^8	4.0

All values are means of three replicates.

weeks for re-fermentation, after that, the number of viable cells was determined 3 d later, which was shown in Table 1. The determination results indicated that the number of viable cells in re-fermented EM bacteria liquid mixed with 0.1% and 0.25% of oligochitosan had achieved the standards; in re-fermented EM bacteria liquid mixed with 0.5% and 1.0% of oligochitosan, the amount of lactic acid bacteria was large but the amount of yeast hadn't achieved the standards.

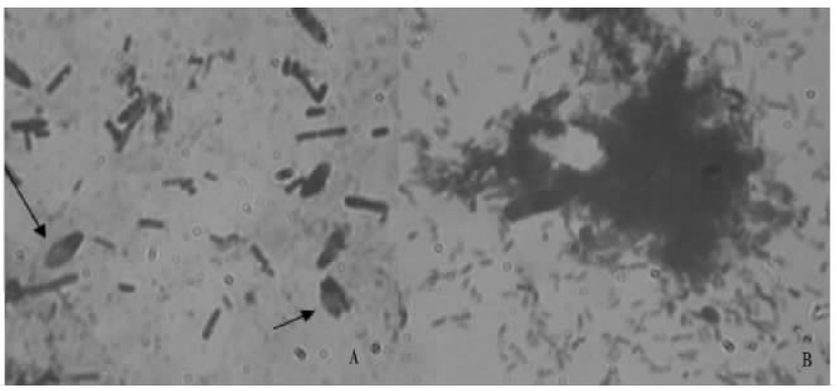
EM bacteria liquid mixed with lactic acid solution and acetic acid solution of oligochitosan was used for re-fermentation after 2 weeks, and the number of viable cells was determined 2 d later, which was shown in Table 2. Experimental data showed that the amount of bacteria in control group and experimental groups had reached the same levels, amount of lactic acid bacteria in re–fermented EM bacteria liquid mixed with lactic acid solution and acetic acid solution of oligochitosan was reduced compared with the control group while the amount of yeast had slightly increased.

Effect of oligochitosan on flatulence of EM bacteria liquid

As can be seen from Table 3 and Fig.7, flatulence in EM bacteria liquid containing more than 0.25% of oligochitosan was significantly inhibited, small bubbles caused by flatulence was significantly reduced or even completely disappeared, which might be due to that some yeast were forced into hibernation resulting from the inhibition of oligochitosan on proliferation of yeast during storage or flocculation effect of oligochitosan[11].

Table 3 Flatulence of EM bacteria liquid with addition of oligochitosan powder

No.	Oligochitosan concentration // %	One week later
1	0.25	Few bubbles
2	1.00	No bubble
3	Control group	Bubbles



A, Yeast flatulence in CK; B, Yeast flocculation in EM bacteria liquid with addition of oligochitosan.

Fig.7 Observation of flatulence in EM bacteria liquid at 1 600 × magnification

Conclusion and Discussion

In this experiment, decrease rate of the number of viable cells in all samples had greatly exceeded that in EM bacteria liquid stored at room temperature, which might be due to the accelerated decline of microorganisms under sealed conditions of high temperature (34 ℃). Another reason was that the addition of oligochitosan had caused flocculation of microorganisms which were sinking to the bottom of the container. In the beginning (1 week), the amount of yeast was gradually reduced while the amount of lactic acid bacteria had increased; however, the amount of lactic acid bacteria had started to decrease with the passage of time.

Addition of 0.25% and 0.5% of oligochitosan was advantageous to extend the shelf life of EM bacteria liquid and maintain the stability of effective microorganisms, lactic acid and acetic acid solutions of oligochitosan had also played a supporting role but effect of acetic acid solution was relatively small. It was found that addition of oligochitosan had certain inhibition effects on flatulence of EM bacteria liquid. The results showed that addition of oligochitosan didn't affect the utilization and re-fermentation effects of EM bacteria liquid. Oligochitosan could be utilized in conjunction with EM bacteria liquid, which had practical application value.

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典型干旱草原区地下水环境质量综合评价

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摘 要 [目的] 对典型干旱草原区的地下水环境质量进行综合评价。[方法] 以内蒙古阴山北麓内陆河流域的达尔罕茂明安联合旗(达茂旗)为研究区,通过污染源调查、实地水质监测分析,采用内梅罗指数法对该研究区地下水环境质量进行综合评价,并对其进行污染成因分析。[结果]达茂旗地下水环境质量处于较差与极差之间。[结论] 该研究为达茂旗地区地下水环境污染的治理奠定了基础。

关键词 干旱草原区;水环境质量;评价;达茂旗

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低聚壳聚糖在 EM 菌液中的溶解及体系稳定性研究

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摘 要 [目的]探讨低聚壳聚糖与 EM 菌的协同效应,为将该类产品应用于动物保健、水产养殖、污水处理等领域提供理论依据。[方法]将不同浓度的低聚壳聚糖添加到 EM 菌液中,并对该 EM 菌液的稳定性进行初步研究。[结果]在 EM 菌液中添加比例适宜的低聚壳聚糖,对 EM 菌液的稳定保存有一定辅助作用,并且对 EM 菌液胀气有一定的抑制作用。低聚壳聚糖的加入对 EM 菌的品质及再发酵效果无明显影响。[结论]该研究表明低聚壳聚糖可与 EM 菌液复配使用,具有实际应用价值。

关键词 低聚壳聚糖;EM 菌液;稳定性

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