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Antifungul Mechanism of Active Substances Produced by *Lactobacillus* Against Yeast

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Research Article

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ABSTRACT

At present, some research work has been done on the antibacterial substances produced by lactic acid bacteria, but its antibacterial mechanism is not yet fully understood. The objective of this study was to investigate the inhibiting mechanism of lactic acid bacteria on yeast. Two strains of *Lactobacillus* ALAC-3 and ALAC-4 being of strong antiyeast ability were studied in the research. Taking *Candida albicans* as the indicator strain, the inhibiting mechanism was explained by determining the effect of the crude extract of active material on the growth curve, membrane permeability, protein expression and interaction with DNA of yeast. The results were showed that inhibitive substance produced by two strains had the influence on the growth of yeast, the logarithmic growth period was shortened and biomass was decreased. The electrical conductivity, soluble protein content and soluble total sugar content in the culture media of yeast were found to increase after treated by inhibiting substances. It indicated that integrity of yeast cell membrane was changed, so all of these were oozed out from yeast cell. The influence on protein expression of yeast was also detected through SDS-PAGE. Some protein expressing bands of yeast appeared deficient or decreased after treatment by inhibiting substance. Ultraviolet spectroscopic techniques showed that antiyeast substance acted on yeast DNA through the electrostatic binding and groove binding.

INTRODUCTION

Food safety is critically related to public health and there is increasing attention in this area in recent years. The emergence of new foodborne disease outbreaks caused by foodborne spoilage and pathogenic bacteria is one of the major food safety challenges [1,2]. During the process of food processing, people use various technical measures to prevent the normal life activities of microorganisms, so that food can be easier to preserve a long time. Which is very convenient to use, and effective method is to add preservatives in food, so the addition of food preservatives are generally used [3]. Along with the development and innovation of biotechnology, people have studied the natural food preservative, which is produced by the use of plant, animal and microbial metabolites as raw material, through the extraction, fermentation or use of various enzymes to produce natural food preservatives. Because of its non-toxic, harmless, safe and gradually get people's attention and vice versa is an important trend of China's future development of preservatives [4].

Yeast fungus pollution and food health relationships: bacteria, yeasts and molds play important role in food spoilage processes. Under normal circumstances, bacteria often have the advantage of yeast and mold, but the yeast genera (genus *Saccharomyces*), also known as sugar yeast, it can be resistant to high concentration of sugar, make syrup, honey and candied fruit and other food fermentation rancidity. In most cases, yeast is good for the human body. But the presence of a large number of yeasts can not only cause food flavor and deterioration, and even to promote the growth of pathogenic bacteria. The yeast has a strong resistance to all kinds of preservatives, ionizing radiation exposure, freezing, etc. It may become the dominant bacteria in food spoilage. At present, some research work has been done on the antibacterial substances produced by lactic acid bacteria, but there are few studies on its antibacterial mechanism. Our aim was to study the effect of antimicrobial substances on the

growth curve of yeast, and its effect on the cell membrane permeability, protein expression and the interaction with DNA, finally to elucidate its mechanism of inhibiting yeast.

MATERIALS AND METHODS

Microorganisms and Chemicals

Lactobacillus: Two strains of ALAC-3 and ALAC-4 which were screened from the traditional fermented food in Inner Mongolia and maintained in punctures of MRS solid medium at 4°C inocula were prepared by 24 h culture in MRS liquid medium at 37°C.

Indicator bacteria: *Candida albicans*, standard strain, the number is 32819, from the China industrial microbial strains preservation management center and maintained in slants of YEPD solid medium at 4°C, inocula were prepared by 36 h culture in YEPD liquid medium at 30°C. All other chemicals used in the study were of analytical grade

Preparation of Crude Extracts of Antimicrobial Substances

The *Lactobacillus* was expanded to the MRS liquid medium (37°C, 24 h), the suspensions were centrifuged at 4000 g for 10 min and supernatant was concentrated to 25 times. Then the appropriate amount of saturated ammonium sulfate solution were added into the concentrated solution and placed at 4°C overnight. The sample was centrifuged (4°C, 8000 gx, 20 min), and discarded the supernatant. The precipitation is configured to be a crude extract of different mass concentrations by using sterile distilled water, mass concentrations were 0.05 g/mL, 0.5 g/mL, 2 g/mL, 4 g/mL, respectively. It was stored at 4°C.

The Study of Growth Curve

The 0.1 mL yeast solution and 0.1 mL crude extract of active material (4 g/mL) were added into 5 mL YEPD liquid medium. To add 0.1 mL yeast solution and equal amount of sterile water into 5mL YEPD liquid medium as blank control. They were incubated at 30 °C for 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36 h, and the absorption value at 600 nm was measured by using an ultraviolet spectrophotometry. Each test was performed in triplicate [5].

Permeability of Cell Membrane

The electrical conductivity, soluble protein content and soluble total sugar content of yeast were examined to express the change of membrane permeability [6]. The yeast solution was added to normal saline, after completely mixed and different concentrations of crude extract of active material were also added into it. To add equal amount of sterile water as blank control, the electrical conductivity was measured in a certain period of time. Each test was performed in triplicate.

The integrity of cell membrane was evaluated by measuring the release of proteins into cell suspension. According to the method used by Lv et al. [7], yeast cells from suspension (100 mL) of yeast were collected by centrifugation for 15 min at 4000 g. Cells were washed three times and re-suspended in PBS (0.1 M, pH7.4). The 100 mL washed suspension were shacked and incubated at 30°C for 4 h in the presence of variable concentrations of crude extract of active material. Then, the suspensions were centrifuged at 4000 g for 5 min. After that, the supernatants were collected, diluted with PBS, and the absorption at 595 nm was measured by using an UV spectrophotometer.

The yeast solution was added to normal saline, after completely mixed and different concentrations of crude extract of active material were also added into it. Then the mixture was treated with 2, 6, 10, 24 h at 30°C, respectively. After that it was centrifuged at 4000 g for 10 min and the supernatants were collected. The Anthrone reagent was added into supernatants and heated in boiling water with 7 min, after standing in dark for 10 min, the absorption at 620 nm was measured by using an UV spectrophotometer, the soluble total sugar content was found in the glucose standard curve. Each test was performed in triplicate.

SDS-PAGE

The yeast solution and 4 mg/mL crude extract of active material were added into 50 mL YEPD liquid medium. To add 0.1 mL yeast solution and equal amount of sterile water into 5 mL YEPD liquid medium as blank control. The mixture was treated for 2, 6, 10, 24 h at 30°C respectively. All samples were centrifuged at 6000 g for 10 min, and then the supernatants were discarded. The cell pellet was rinsed and resuspended in normal saline. Before the SDS-PAGE analysis, the Yeast cells suspension was disrupted by ultrasonic wave for 30 min and centrifuged at 6000 g for 10 min. Discarded the supernatant, the sterile water was added into precipitates and mixed evenly. The buffer was added into samples according to the volume ratio of 1:3. The mixture was boiled for 5 min and cooled on ice. Then, 20 μL each sample was collected for the SDS-PAGE analysis. After electrophoresis, the gel was stained with coomassie brilliant blue R-250 and then decolorized to obtain the separated protein bands.

Interaction with DNA of Yeast

The yeast was inoculated into YEPD liquid medium (30°C, 36 h).Then the yeast solution was centrifuged at 4000 xg for 15 min. After that, the precipitates were collected. The yeast genomic DNA was extracted by glass bead method. The concentration of DNA is 398.0ng/μl. Purity of the DNA was checked by monitoring the absorbance ratio A260 /A280 in the range of 1.8–2.0, indicating that the DNA was sufficiently free from protein [8,9]. The DNA and different concentrations of crude extract of active material were interacted in Tris-HCl buffer (0.1 M, pH 7.4). The UV-VIS absorption spectra at range of 220-320 nm was measured by using an UV spectrophotometer equipped with a conventional quartz cell of 1.0 cm path length.

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RESULTS AND DISCUSSION

Effect of the Crude Extract of Active Material on the Growth Curve of Yeast

The time-kill data of two strains were displayed in **Figure 1.** The blank control is the normal growth curve of yeast at 30°C. After addition of inhibiting substances, the OD value of the same growth time was lower than the control. Meanwhile the logarithmic growth period was shortened and biomass was decreased. It showed that the growth of yeast was affected by the inhibiting substances. And effect of antimicrobial substances produced by ALAC-4 on yeast growth was greater than ALAC-3.

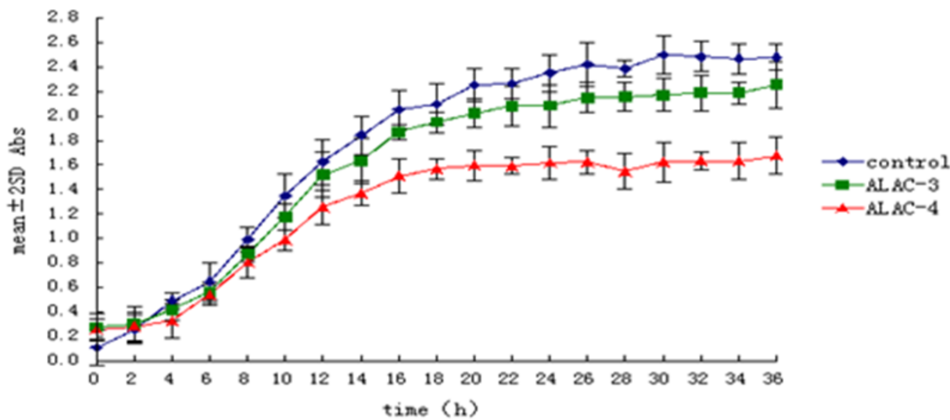

Figure 1. Effect of the crude extract of active material on the growth curve of yeast.

Effect of the Active Material on Membrane Permeability

Electric conductivity of yeast broth was examined to express the permeability changes of bacteria cell membranes. Results **(Figures 2 and 3)** showed that the electric conductivity of yeast suspension was lower than the control when the concentration of antimicrobial substance was 0.05 g/mL. The reason might be that the antimicrobial substance didn't destroy the cell membrane, but combined with the outer surface of the cell membrane by electrostatic bonding, which leaded to the decrease of charged ions and then reduce the electrical conductivity of the medium. The electric conductivity was higher than the control when the concentration of antimicrobial substance was 2 g/mL and 4 g/mL. The electric conductivity increased rapidly in respond to increasing levels of antimicrobial substance during the first few minutes. After that, the growth tends to slow down. The results indicated that antimicrobial substance could increase the permeability of cell membrane causing cellular leakage. And furthermore, the yeast suspension treated by inhibiting substance coming from ALAC-4 showed higher electric conductivity than that of ALAC-3.

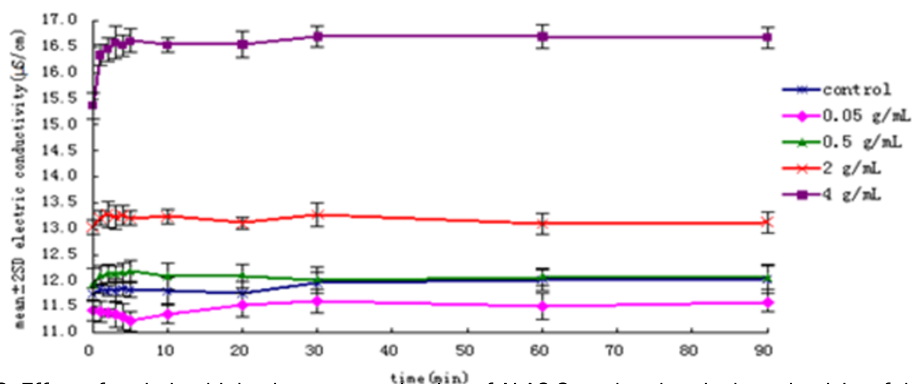

Figure 2. Effect of antimicrobial substance production of ALAC-3 on the electrical conductivity of the yeast.

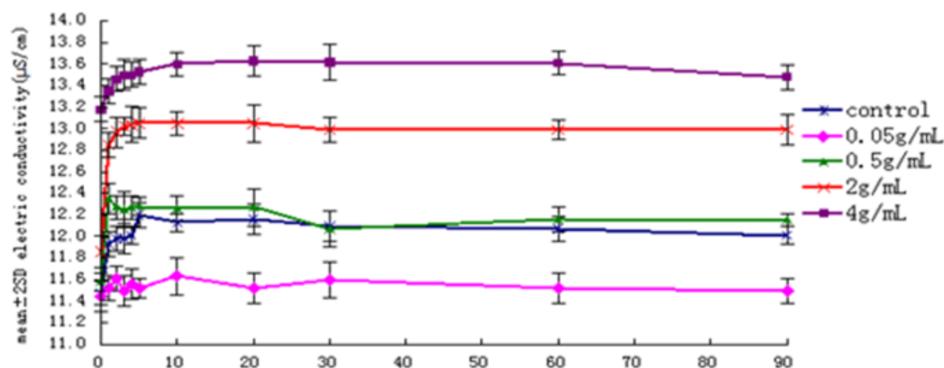

Figure 3. Effect of antimicrobial substance production of ALAC-3 on the electrical conductivity of the yeast suspension.

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Information on the release of cell constituents can reveal the integrity of cell membrane. It was evident in the study that soluble proteins were released into cell suspension and their levels increased after contacting with antimicrobial substance **(Figure 4).** The content of soluble protein in yeast suspension was higher than control.

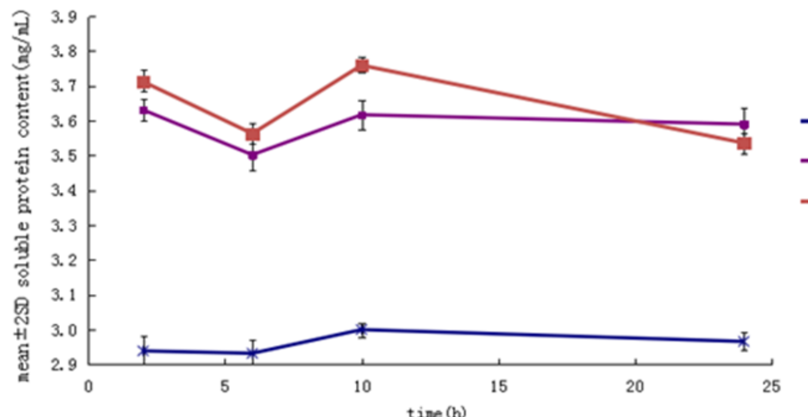

Figure 4. Effect of antimicrobial substance production on the soluble protein of the yeast suspension.

In the case of two strains of bacteria, the change was consistent when the yeast was reacted with the same concentration of antimicrobial substances. The content of soluble protein was significantly decreased from 2 h to 6 h, it means that the antimicrobial substance may inhibit the rate of yeast protein synthesis. And then the soluble protein content was increased obviously from 6 h to 10 h. It was followed by a steady state after 10 h. During the period, the permeability of yeast cell membrane was destroyed and the content of soluble protein in media was increased. Meanwhile the changes of total soluble sugar content were found **(Figure 5).** The soluble sugar content was increased comparing with the control after addition of antifungal substance.

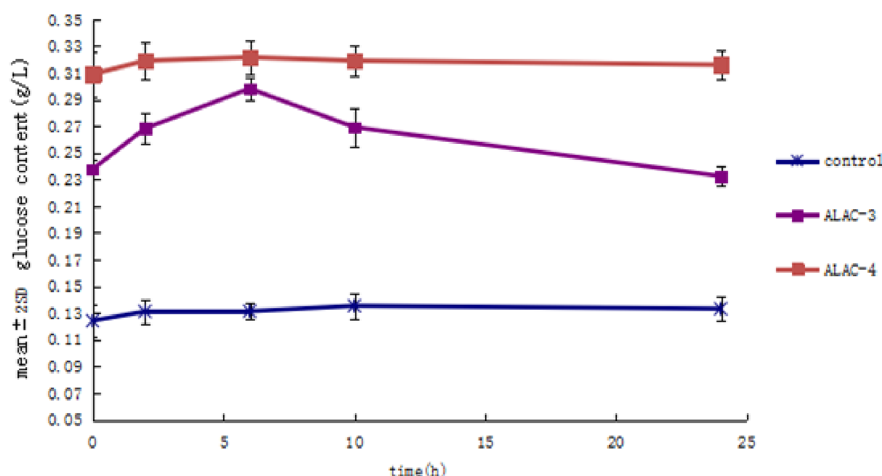

Figure 5. The changes of total soluble sugar content.

The results indicated that the integrity of yeast cell membrane had been destroyed after exposure to antimicrobial substance, which can be observed by scanning electron microscope and transmission electron microscope (data not show), so the soluble proteins and intracellular carbohydrate were released into cell suspension. This may be one of the evidences that inhibiting substance consequently leaded to death of yeast cell.

SDS-PAGE

Protein plays an important role in the life activity of microbial cells. Protein electrophoresis bands of treated yeast were significantly different from the controls. After added the active material, the protein bands of yeast appeared to change. With the extended treatment time, the bands were fainter, and some even disappeared, such as band A, B, C in **Figure 6**, and band A, B, C, I in **Figure 7.** All bands in **Figure 7** were fainter than the previous one. It implied that the active material had a remarkable effect on yeast proteins expression either by destroying them or by inhibiting their synthesis, and then resulting in their death. Zeng, X reported the similar result [10].

UV Absorption Spectra of the Interaction between DNA and Antiyeast Substance

For the agents which bind to DNA, intercalation, groove binding and electrostatic binding are the three primary binding modes [11,12]. The binding of inhibiting substance to DNA had been characterized through hypochromism and hyperchromism in the absorption spectra [13]. When inhibiting substance was intercalated between base pairs of nucleic acids, a red shift and

hypochromism were observed at the absorption maximum [14]. The absorption spectra showed that both ALAC-3 and ALAC-4 had absorption peaks at 269 nm, and the absorption peak decreased significantly without any band shifted with the amount of antiyeast substance increased **(Figures 8 and 9).** So the binding mode was not the intercalative binding. Some researchers investigated the binding mode between small molecules and DNA using the UV absorption spectra of DNA. In general, electrostatic binding can neutralize the negative charged phosphate groups of DNA. As a consequence, DNA contracts and the hypochromic effect appears [15]. This indicated that the binding mode of antiyeast substance to yeast DNA might be electrostatic binding.

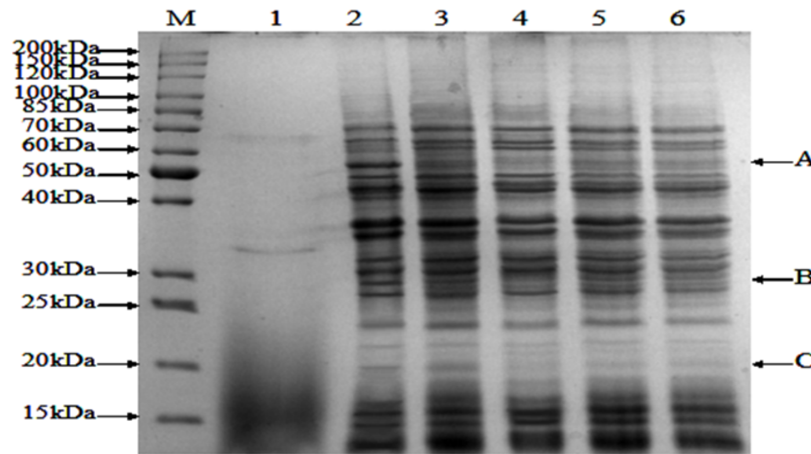

Figure 6. Effect of antimicrobial substance production of ALAC-3 on the protein expression of yeast M: Marker#26614; 1: Crude extract of active material produced by ALAC-3; 2: Yeast suspension; 3-6: add the crude extract of active material into yeast for 4, 8, 12, and 24 h.

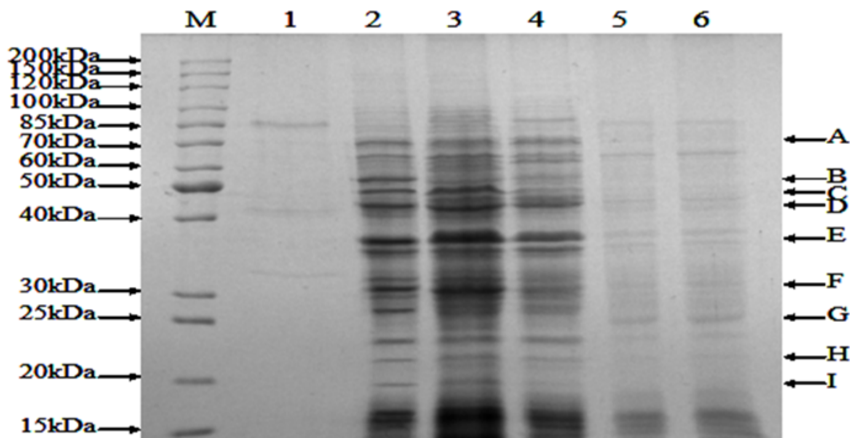

Figure 7. Effect of antimicrobial substance production of ALAC-4 on the protein expression of yeast M: Marker#26614; 1: Crude extract of active material produced by ALAC-4; 2: Yeast suspension; 3-6: add the crude extract of active material into yeast for 4,8,12 and 24 h.

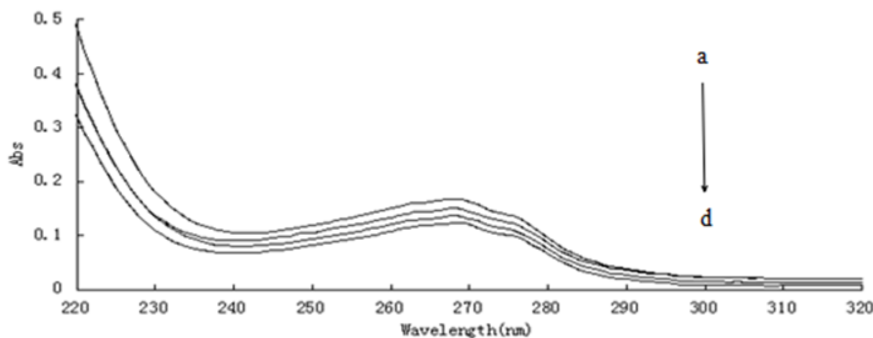

Figure 8. UV absorption spectra of the interaction between DNA and different concentrations of antiyeast substance produced by ALAC-3. Content (anti yeast substance)/(X10^{-3} g.mL^{-1}) (a-d):0,0.003,0.006,0.009, respectively.

Furthermore the absorption peak was increased with the extension of interaction time using same concentration antiyeast substance to react **(Figures 10 and 11).** The application of absorption spectroscopy can give useful information in DNA-binding. In the absorption spectrum, hyperchromism derives from damage to the DNA double-helix structure .The hyperchromism was observed without any band shift at 269 nm with the addition of antiyeast substance. The reason might be that antiyeast substance was bound to yeast DNA by groove binding, and the space conformation of DNA became loose, so a hyperchromism was observed. It showed that the double helix was affected by the binding between antiyeast substance and yeast DNA.

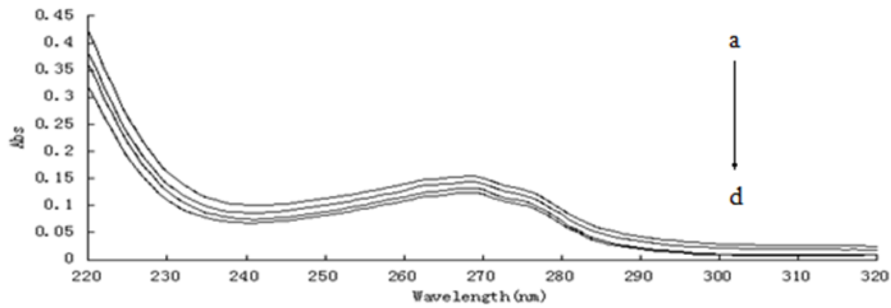

Figure 9. UV absorption spectra of the interaction between DNA and different concentrations of anti-yeast substance produced by ALAC-4. Content (anti yeast substance)/(X10^{-3} g.mL^{-1}) (a-d):0,0.003, 0.006,0.009, respectively.

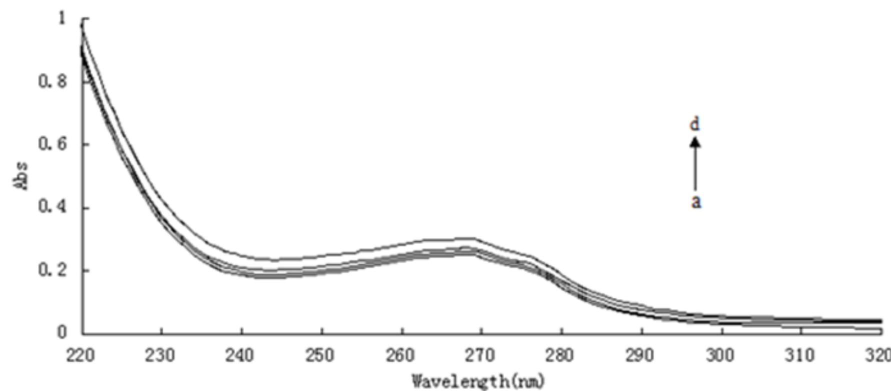

Figure 10. UV absorption spectra of the interaction between DNA and antiyeast substance produced by ALAC-3 at different time. a-d: 3 h, 6 h, 12 h, and 24 h.

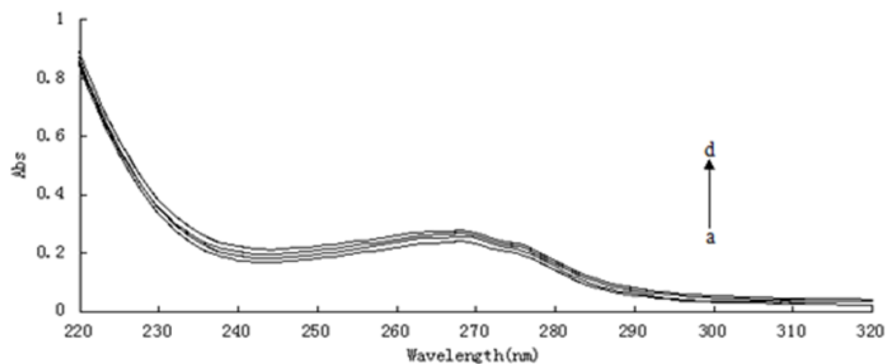

Figure 11. UV absorption spectra of the interaction between DNA and antiyeast substance produced by ALAC-4 at different time. a-d: 3 h,6 h,12 h and 24 h.

CONCLUSION

In this research, the inhibiting mechanism of antiyeast substances produced by *lactobacillus* was studied. The results showed that antiyeast substance had the effect on the growth curve of yeast. The logarithmic growth period of yeast was shortened and biomass was decreased. The electrical conductivity, soluble protein content and soluble total sugar content in culture media of yeast increased after treated by inhibiting substances. It indicated that integrity of yeast cell membrane was damaged, so the electrolytes, soluble protein and soluble total sugar in yeast cell were oozed out. And the active substances coming from ALAC-4 had a greater effect on permeability of yeast cell membrane. The protein expression in yeast was also influenced by inhibiting substance. The expressing amounts of some proteins of yeast were reduced or deficient. The interaction of antiyeast substance and yeast DNA was studied by UV spectroscopic techniques. The ultraviolet absorption value was decreased significantly and hypochromism were observed after adding antimicrobial substances to yeast DNA. With the extension of treatment time, the ultraviolet absorption value was increased and hyperchromism were observed. The results showed that antiyeast substance interacted with yeast DNA through the electrostatic binding and groove binding.

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REFERENCES

1. Arques JL, et al. Inactivation of gram-negative pathogens in refrigerated milk by reuterin in combination with nisin or the lactoperoxidase system. Eur Food Res Technol. 2008;227:77-82.

2. Belma A and Nihal Y. In vitro antimicrobial activity of essential oil from endemic Origanum minutiflorum on ciprofloxacin-resistant Campylobacter spp. Food Chem. 2007;107:602-606.

3. Li Wang, et al. Application and development trend of food preservatives in China. J Food Safety Food Qual. 2011;2:83-87.

4. Sanli Shi, et al. Application status and future development trend of food preservatives in China. Food Res Dev. 2008;29:157-161.

5. Yong MA and Ya TU. Determination of growth curve of oil producing yeast in high concentration culture by Spectrophotometry. Northern horticulture. 2013;8:116-118.

6. Diao WR, et al. Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of fennel(Foeniculum vulgare Mill.). Food Contr. 2014;35:109-116.

7. Lv F, et al. In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. Food Res Int. 2011;44:3057-3064.

8. Shahabadi N, et al. DNA interaction studies of a platinum(II) complex, PtCl(2)(NN) (NN=4,7-dimethyl-1,10-phenanthroline), using different instrumental methods. Spectrochim Acta A Mol Biomol Spectrosc. 2009;72:757-761.

9. Tong C. et al. Investigation on the toxic interaction of typical plasticizers with calf thymus DNA131. J Lumine. 2011;2133.

10. Zeng X, et al. Studies on disinfection mechanism of electrolyzed oxidizing water on E. coli and Staphylococcus aureus. J Food Sci. 2010;75:253-260.

11. Erkkila KE, et al. Recognition and reaction of metallointercalators with DNA. Chem Rev 1999;99:2777-2796.

12. Shi Y, et al. Interaction between DNA and Microcystin-LR studied by spectra analysis and atomic force microscopy. Biomacromolecules. 2011;12:797-803.

13. Wu HL, et al. Spectral studies of the binding of lucigenin, a bisacridiniumderivative, with double-helix. DNA Anal Bioanal Chem. 2002;373:163-168.

14. Li FH, et al. Synthesis, characterization and biological activity of lanthanum(III) complexes containing 2-methylene-1,10-phenanthroline units bridged by aliphatic diamines. J Inorg Biochem. 2006;100:36-43.

15. Lv F, et al. 2011. In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. Food Res Inter. 2011;44:3057-3064.