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Full Length Article

Antimicrobial Spectrum and Preliminary Screening of Substance from Strain HRH317 of *Bacillus amyloliquefaciens*

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Abstract

Antimicrobial spectrum and substance of Strain HRH317 of *Bacillus amyloliquefaciens* were studied in this paper. The antimicrobial substance was determined as bacteriocin by eliminating acid, H_2O_2 and enzyme treatment experiment, meanwhile, the crude proteins were gained from the fermentation supernatant by fractional precipitation of ammonium sulphate and purified by sequential column chromatography using DEAE Sepharose Fast Flow and Sephadex G-200 as column filling matrix. The results indicated that a kind of antimicrobial protein found with molecular weight of 36 kDa by SDS-PAGE. The results showed that antimicrobial spectrum was widely by detecting the antimicrobial substance activity with oxford-cup tests, and especially inhibition effect was good for the fruit spoilage mould. Therefore, the antimicrobial substances have wide development foreground and value. © 2020 Friends Science Publishers

Keywords: *Bacillus amyloliquefaciens*; Antimicrobial spectrum; Screening; Antimicrobial protein; Antimicrobial peptide

Introduction

Recently, the research of microbial antimicrobial substance development has become a hot field of microbiological research. Since 1945, Johnson reported *Bacillus subtilis* antimirobial substances, various antimicrobial substances produced by microorganisms caused widespread concern (Maget and Peypoux 1994). *Bacillus* is a non-pathogenic bacteria existing widely in nature, can produce a variety of antimicrobial substances, most of which are polypeptide, mainly against gram positive bacteria and some gram negative bacteria, fungi and yeast (Magnusson and Schnürer 2001). *B. amyloliquefaciens* and *B. subtilis* affinity was high, in recent years, it has been reported that a series of metabolic products can be produced in the growth process itself, these metabolites with active inhibition of fungi and bacteria (Wright *et al.* 2001). Many scholars have studied on *B. amyloliquefaciens*, the bacteria have the advantages of easy separation, easy culture. Identification methods consist of the inhibitory effect of screening, the individual morphology, physiological and biochemical characteristics, determination of fatty acid, sequence analysis of 16S rDNA homology, HPLC-MS analysis and others. Extraction and separation method of antimicrobial substances are mainly macro porous resin method, acid-alkaline precipitation, ammonium sulfate precipitation, chromatography *etc*.

A kind of oligopeptide was found in *B. amyloliquefaciens* 23-7A. The biochemical activity, protein, amino acid sequence and enzyme activity was similar with other bacterial PepF (Shiou-Huei *et al.* 2006). One *B. amyloliquefaciens* strain were isolated from probiotics of yoghurt. The sterile supernatant cultured during one night, can produce inhibition on bacteria Lester. At the same time, *vaginal bacteria Gardiner* and no *Streptococcus lactis* were inhibited in the clinical trial; its effective antimicrobial substances were bacteriocin (Sutyak *et al.* 2008). *B. amyloliquefaciens* DGA14 was separated from banana surface and it found that has inhibition against the banana crown rot-causing pathogens (Alvindia and Natsuaki 2009). The *B. amyloliquefaciens* LBM 5006 is separated from Brazil forest in the Atlantic, it can inhibit *Aspergillus* spp., *Fusarium* spp., and *Bipolaris sorokiniana* (Lisboa *et al.* 2006). The papaya was treated by the *B. amyloliquefaciens* binding 1-MCP, the results indicated that anthracnose soft rot disease and were inhibited effectively (Osman*et al.* 2010). Endogenous *B. amyloliquefaciens* strain TB2 genome as the template, the bacteria ß-1, 4- endoglucanase gene ORF was cloned. Blast homology analysis showed that, the sequence homology was same as 1 strains of *B. subtilis* (strain M 28332) cellulase nucleotide sequence homology (98%) aligned with the GenBank, and the amino acid homology reach to 98% (Fan *et al.* 2008).

Antimicrobial substances produced by microorganisms including antibiotics, bacteriocin,

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antimicrobial protein, hydrogen peroxide and organic acids *etc*. Therefore, it was necessary to exclude the interference factors for the screening of bacteriocin producing strains and inhibition of bacteriocin to be determined. There were some methods such as eliminating acid, eliminating hydrogen peroxide, and protease detection (Leelasuphakul *et al.* 2008). Bacteriocins were protein or polypeptide produced by the bacteria metabolic process, there was some inhibition effect of certain microbial growth activity, and some degradation by protease. Bacteriocins have some advantages of non-drug-resistant, no residue, and inhibition of pathogenic bacteria (Garneau *et al.* 2002; Wang *et al.* 2013). So, it has a broad application prospect in food, medical, health and veterinary *etc*. (Neville and O'Toole 2010; Maldonado *et al.* 2010).

The strain HRH317 of *B. amyloliquefaciens* was isolated from corn fields after harvest around Shanxi Agricultural University by professor Hao Lin's team of Shanxi Agricultural University in 2011. The genomic DNA of strain H317 was extracted by the kits method, then bacterial universal primers were used as amplification primer, 16S rDNA sequence was amplified by PCR, and the sequence and homology were compared. The results showed that 16S rDNA sequence of HRH317 were same as homology of *B. amyloliquefaciens*, reach to 99%-100%. At the same time the phylogenetic tree of 16S rDNA sequences were drawn. Comprehensive comparison, HRH317 was identified as *B. amyloliquefaciens*. The study found that the strain has excellent antimicrobial activity, its antimicrobial spectrum was widely, not only can inhibit the gram negative and gram positive bacteria, but also can inhibit the yeast and mold, it was a good kind of resource with the development in the microorganism. At present, the antimicrobial spectrum and antimicrobial substances component of HRH317 were studied, to lay a good foundation and theoretical support for the further development and utilization.

Materials and Methods

Microorganisms and medium

Strain HRH317 of *B. amyloliquefaciens*, Penicillium: provided by biology engineering experiment of Shanxi Agricultural University. Other indicator strains and number are given in Table 1.

Beef extract peptone medium (used by the tested bacteria and indicator bacteria) (Hao 2001): beef extract 0.5 g, peptone 1.0 g, NaCl 0.5 g, agar 1.5 g, water 100 mL, pH 7.2, 0.1 MPa, sterilization 20 min.

Mould medium (Hao 2001): potato 200 g, were peeled and cut into the water 1000 mL and boiled for 30 min, filtrated by double-layer gauze, glucose 20 g, agar 20 g, 0.075 MPa, sterilization 20 min.

Yeast medium (Hao 2001): peptone 10 g, maltose 20 g, yeast extract 5 g, agar16 g, distilled water 1000 mL.

Liquid activation medium (Hao 2001): beef extract 0.5 g peptone 1.0 g, NaCl 0.5 g, water 100 mL, 0.1 MPa, sterilization 20 min.

Medicine and reagent: 1 mol/L HCl, 1 mol/L NaOH , catalase, protease K, ammonium sulphate, Tris buffer solution, DEAE Sepharose Fast Flow, sephadex G-200, Coomassie brilliant blue G- 250.

Antimicrobial activity detection

Oxford-cup tests (Hao 2001; Schnürer and Magnusson 2005), The solid medium sterilized 15 mL were added into the culture dish sterilized, the spore suspension of indicator bacteria 0.2 mL were added into the culture dish by micro pipette after solidification, and were speeded evenly with glass spreading rod. The oxford-cup were put into the culture dish equidistantly when the suspension was absorbed, the fermentation supernatant of strain HRH317 0.2 mL were injected into each oxford-cup and put in the refrigerator at 4°C for 24 h and then put in the incubator at 30°C for 3-5 days. Observe the inhibition circle.

Antimicrobial spectrum detection

Gram positive bacteria, gram negative bacteria, yeast and mould used as indicator bacteria were detected by the antimicrobial substance of the strain HRH317 of *B. amyloliquefaciens*. The antimicrobial spectrum could be determined through the inhibition effect (Quiroga *et al.* 2001; Flynn *et al.* 2002; Gong *et al.* 2006).

Preparation method of bacteria suspension: the inclined bacteria were picked by sterilized inoculating loop into the liquid medium 30 mL in the shaker incubator at 37°C, 160 r/min for 24 h.

Preparation method of yeast suspension: the same to bacteria, at 30°C, 150r/min for 48 h.

Preparation method of mould spore's suspension: the inclined mould were picked by sterilized inoculating loop put into the normal saline 5 mL, concentration was made into 1.5×10^6 per mL.

Every kind of indicator bacteria experiment repeated 3 times, measured the diameter of inhibition circle and calculate the average value.

Antimicrobial substance screening and analysis

Eliminating acid

The inhibition effectiveness of fermentation supernatant to the indicator bacteria may be due to the acid produced by strain HRH317. Fermentation liquid was centrifuged under the conditions of 5000 r/min, 20 min, added 1 mol/L HCl and 1 mol/L NaOH into fermentation supernatant to adjust the pH 7 and operated the inhibition experiment by oxford-cup tests (Joshi *et al.* 2008).

Eliminating hydrogen peroxide

Antagonism could inhibit the microorganism by the hydrogen peroxide produced by it. Fermentation liquid were

centrifuged under the conditions of 5000 r/min, 20 min, added the catalase into fermentation supernatant in the bain-marie at 37℃ for 4 h and operated the inhibition experiment by oxford-cup tests after eliminating hydrogen peroxide (Li *et al.* 2006).

Protease detection

Fermentation liquid was centrifuged under the conditions of 5000 r/min, 20 min, added 1 mol/L HCl and 1 mol/L NaOH into fermentation supernatant to adjust the pH 7 and added catalase and protease K in the bain-marie at 37℃ for 2 h, bacteria was used as indicator bacteria and operated the inhibition experiment by oxford-cup tests after eliminating hydrogen peroxide (Kamel *et al.* 2012).

SDS-PAGE detection

Precipitation of ammonium sulphate (Matynia *et al.* 2005): The fermentation supernatant was filtrated by 0.22 μm bacteriological filter, the cell-free cultural filtrate was gained. The ammonium sulphate were added into the filtrate to the saturation of 50% at 4℃ left to stand 2 h, the sediment was collected under the centrifuge conditions of l0000 r/min at 4℃ for 30 min. The sediment was dissolved by pH 6.8 Tris buffer solution of 1/10 of sediment volume and put into the bag filter of the same concentration of Tris buffer solution one night. The crude protein from sediment were re-dissolved and treated by DEAE ion exchange chromatography and Sephadex G-200 column in sequence (Queiroz 2004).

SDS—PAGE: According to literature (Idriss *et al.* 2002). spacer gel 4%, separation gel 12%, standard protein produced by Shanghai Biology Chemical Institute. The antimicrobial protein component was detected by oxford-cup tests with three repeated experiments.

Results

Antimicrobial spectrum of fermentation supernatant

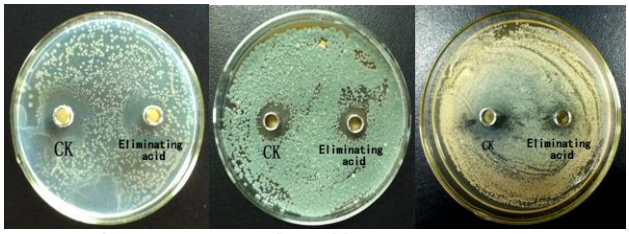
Antimicrobial spectrum of the fermentation supernatant of strain HRH317 of *B. amyloliquefaciens* to common microorganisms were showed in Table 1.

Table 1 indicated that the antimicrobial substances of fermentation supernatant consist of three kinds of microorganisms, such as bacteria, yeast and mould, so the strain HRH317 of *B. amyloliquefaciens* has widely antimicrobial spectrum.

Antimicrobial substance screening and analysis

Eliminating acid

The strain HRH317 of *B. amyloliquefaciens* in the shaker incubator were cultured at 37℃, 160 r/min for 24 h, fermentation liquid pH was 6.8. Acid substances were



1-a bacteria 1-b mould 1-c yeast

Fig. 1: Inhibition activity after eliminating acid
Left tube: the control, right tube: the treatment

eliminated still have antimicrobial activity, and the diameters of inhibition bacteria circle were nearly the same (Fig. 1), the results showed that inhibition effective were not the acid substances come from the strain HRH317.

Eliminating hydrogen peroxide

Adding catalase treatment was compared to the non-catalase treatment, the results showed that inhibition activity of CK and treatment group were the same nearly for *B. coagulans* 63501-2aq (Fig. 2a). For *Penicillium* (Fig. 2b) and yeast (Fig. 2c), the inhibition activity of treatment group was disappeared completely. The results showed that antimicrobial substances of strain HRH317 were not only consist of hydrogen peroxide that inhibit the bacteria, mould and yeast, but also include non-hydrogen peroxide substances that inhibit bacteria.

Protease detection

The protease treatment could decide the antimicrobial substance were protein or polypeptide. The inhibition bacteria circle disappeared mostly when fermentation liquid of strain HRH317 was combined with protease K and the CK had an obvious inhibition activity (Fig. 3). The results showed that non hydrogen peroxide substances produced by the strain HRH317 of *B. amyloliquefaciens* belonged to protein or polypeptide.

Shown experiments, the strain HRH317 of *B. amyloliquefaciens* when fermented could produce two kind of antimicrobial substances, as follows hydrogen peroxide that inhibition spectrum widely and protein and polypeptide that antibacterial selectively.

SDS—PAGE purity detection

From Fig. 4 the results showed that the crude proteins that inhibition activity was gained when the fermentation supernatant was precipitated fractionally by ammonium sulphate, and consist of different kind of protein components (column 1). Meanwhile, indicated that strain HRH317 include many kinds of different molecular weight proteins except for antimicrobial protein. The crude protein from sediment were re-dissolved and treated by DEAE ion

Table 1: The antimicrobial spectrum of substance from HRH317

Indicator bacteria	Source	Diameter of inhibition bacteria circle (average value:mm)
Bacillus spp. H108	Microbiology Laboratory of SXUCM (ML of SXUCM)	26.5 ± 1.20
B. coagulans 10144	CICC	18.0 ± 0.51
B. coagulans 63501-2aq	CMCC	14.5 ± 0.23
Escherichia coli 44102-3a11	CMCC	13.0 ± 1.30
Staphlococcus aureus 26003-5a1	CMCC	16.0 ± 1.06
Saccharomy cescerevisiae H226	(ML of SXUCM)	14.0 ± 0.92
Rhodotorula H215	(ML of SXUCM)	15.5 ± 0.86
Saccharomyces ellipsoideus H222	(ML of SXUCM)	14.5 ± 1.12
Alternaria kikuchiana	Isolate from pear, (ML of SXUCM)	21.5 ± 0.98
Plasmopara uiticola R6	Isolate from grape, (ML of SXUCM)	18.5 ± 1.35
Aspergillus niger	(ML of SXUCM)	20.0 ± 1.28
Penicillium	(ML of SXUCM)	19.5 ± 1.03
Fusarium moniliforme	(ML of SXUCM)	18.5 ± 0.98
F. graminearum	(ML of SXUCM)	16.5 ± 0.75

2-a bacteria 2-b mould 2-c yeast

Fig. 2: Inhibition activity after adding catalase
Left tube: the control, right tube: adding catalase

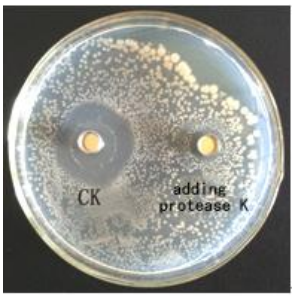


Fig. 3: Inhibitory activity after adding protease K
Left tube: the control, right tube: adding protease K

exchange chromatography, there were many different protein components of peak 2 (column 2). Furthermore, the peak 2 from DEAE were purified by Sephadex G-200 column, an obvious single band were obtained and determined as antimicrobial protein that molecular weight was 36 KD (column 3). Finally, the antimicrobial protein component was detected by oxford-cup tests, the antimicrobial activity effect of three repeated experiments were good to the indicator strain in Fig. 5.

Discussion

The bacterium *B. amyloliquefaciens* is a kind of biological control resource, it has strong reproductive capacity, easy industrialization production, harmless to human and animal, it also causes no pollution to the environment so on, has become a hot point of research and application after the

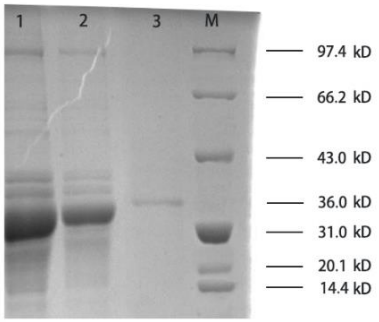


Fig. 4: SDS-PAGE spectrum of antimicrobial protein
Note:1. Ammonium sulfate-precipitated protein;2. DEAE peak 2;
3. Sephadex G-200 peak 2 M.Marker

Fig. 5: Antimicrobial activity detection of antimicrobial protein component

application of the *B. subtilis* in recent years (Sowanpreecha et al. 2018). The small molecular weight of antibiotics and antimicrobial proteins or peptides and other active substances were produced by *B. amyloliquefaciens* which can inhibit various plant pathogenic bacteria, play an important role in the control of food preservation, plant disease. In biological control, *B. amyloliquefaciens* can be produced as biological pesticide or fertilizer, and has wide application prospect. In this experiment,1 strain of antagonistic bacteria strain HRH317 were isolated from maize fields after harvest, through the detection, it was found that *B. amyloliquefaciens* HRH317 fermentation liquid with broad antimicrobial spectrum characteristics, can

inhibit a variety of bacteria, yeasts and molds, especially has good inhibition for the mold caused fruit spoilage.

The antimicrobial substances were studied by the experiment of identification method of producing bacteriocin, the results showed that the antimicrobial substances include hydrogen peroxide and proteins or polypeptides. The fermentation liquid still has antimicrobial activity after eliminating acid and hydrogen peroxide, and then the antimicrobial activity disappeared by the protease treatment, therefore, the antimicrobial substances were determined as protein, furthermore, the *B. amyloliquefaciens* HRH317 were determined as bacteriocin producing strains. The antimicrobial activity was significantly enhanced through the ammonium sulfate precipitation, the dialysis and concentration. Furthermore, the crude proteins were purified by sequential column chromatography using DEAE Sepharose Fast Flow and Sephadex G-200 as column filling matrix. The antimicrobial protein of molecular weight 36 KD were gained through SDS - PAGE detection. At present, the usually methods of isolation and purification of antimicrobial protein were ammonium sulfate precipitation and gradually chromatography purification. But there were small amounts of heavy metal ions, a sensitive effect on protein thiol, and this method often requires a longer time and more equipment. In order to reduce the extraction of protein loss, HCl precipitate can be used to extract the antimicrobial crude protein. Hang get stable relatively antimicrobial protein by above methods (Zhang and Zhang 2010). The molecular weight of antimicrobial protein 40 kD were extracted by *B. amyloliquefaciens* MET0908 to control anthracnose (Kim and Chung 2004).

The antimicrobial protein of separation and purification has advantages as follows good safety, moderate molecular weight, good stability, a widely antimicrobial spectrum and *etc*. There will be good prospect in food preservative and new antimicrobial agent development. The mechanism of antimicrobial protein and gene expression of *B. amyloliquefaciens* HRH317 needs further study.

Conclusion

The results of this study indicate that antimicrobial spectrum was widely by detecting the antimicrobial substance activity with oxford-cup tests and especially inhibition effect was good for the fruit spoilage mould. The antimicrobial substance was determined as bacteriocin by eliminating acid, H_2O_2 and enzyme treatment experiment, meanwhile, the crude proteins were gained from the fermentation supernatant by fractional precipitation of ammonium sulphate, and purified by sequential column chromatography using DEAE Sepharose Fast Flow and Sephadex G-200 as column filling matrix. Finally the antimicrobial subtance was a kind of antimicrobial proteins found that molecular weight was 36 kilo Dalton by SDS-PAGE.

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