Isolation and Antimicrobial Spectrum of New Bacteriocin From Lactobacillus rennanquilfy WHL 3

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ABSTRACT

Production of antimicrobial substances is often regarded a priori as an important trait in the context of bacterial fitness but also in terms of probiotic efficacy. Several probiotic bacteria produce a variety of antimicrobial compounds viz. short chain fatty acids, Hydrogen peroxide, Nitric oxide, Bacteriocins. Bacteriocins are peptide or protein complexes showing antibacterial activity against closely related species. These antimicrobial agents are gaining more and more attention as an alternative therapeutics not only in pharmaceutical but also as a preservative in food industries. In present study, bacteriocin production was carried out by Lactobacillus rennanquilfy WHL 3 isolated from whey and identified by 16s rRNA sequence analysis. This newly isolated strain showed antibacterial activity against several Gram positive and Gram negative bacteria. To obtain maximum bacteriocin yield, medium was optimized with various fermentation conditions like carbon and nitrogen source, Growth factors, pH and incubation temperature. An isolate WHL 3 showed the maximum bacteriocin production in modified MRS medium containing 1% Glucose, 1% Peptone, and 0.8% Yeast extract. Maximum bacteriocin production was observed at 40°C temperature at pH 6.0. Lactobacillus rennanquilfy WHL 3 is capable of producing bacteriocin at wide range of temperatures and hence it is an ideal strain for bacteriocin production for food and pharmaceutical industries.

Keywords: Antimicrobial activity, Bacteriocin, Lactobacillus rennanquilfy WHL 3, Probiotics.

INTRODUCTION

Probiotics are defined as, “live microorganisms which when consumed in adequate amounts, confer a health benefit on the host.” 1 The majority of probiotics in use today include species of Lactic Acid Bacteria (LAB), including Lactobacilli as well as Bifidobacterium, non-pathogenic Escherichia coli, Bacilli and Yeasts such as Saccharomyces boulardii.

Several mechanisms of probiotic action have been described, the most common relating to their abilities to strengthen the intestinal barrier, to modulate the host immune system, and to produce antimicrobial substances. 2 Indeed, the production of antimicrobial substances is often regarded a priori as an important trait in the context of bacterial fitness but also in terms of probiotic efficacy. Several probiotic bacteria produce a variety of antimicrobial compounds viz. Short chain fatty acids, Hydrogen peroxide, Nitric oxide, Bacteriocins., that may enhance their ability to compete against other GI microbes and which could potentially inhibit pathogenic bacteria. 3,4

Bacteriocins are protein or protein complexes produced by bacteria and have antimicrobial activity against closely related species and various Gram positive and Gram negative bacteria including food spoilage bacteria and pathogens. 5 Bacteriocins are in general cationic (i.e. they contain an excess of lysyl and arginyl residues) amphipathic molecules composed of 12 - 45 amino acids residues. They are usually unstructured in aqueous solution, but have the propensity to form α- helical structure when exposed to structure promoting solvents such as trifluoroethanol or when mixed with anionic phospholipid membranes. 6 Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which nisin, diplococci, acidophilin, bulgarican, helveticins, lactacins, lactolin and plantaracins are the important bacteriocins. 7- 8 Nisin is the only bacteriocin commercially available. 9 Other bacteriocins of Lactobacilli have been reported to be effective against closely related species of Lactobacillus and therefore considered as potential natural food preservative. However, studies relating to the antibacterial properties of these organisms are limited and not fully exploited for use. 10, 11

The current work aimed to isolate bacteriocin producing Lactic Acid Bacteria from whey sample, Identification of strains by 16s rRNA sequencing method, production of bacteriocin and determination of antimicrobial activity against common pathogens.

MATERIALS AND METHODS

Chemicals and Media

Analytical grade chemicals were obtained from Qualigenes, Thomas baker and SD Fine India, while media were obtained from Hi-media, India.

Test Microorganisms

The test microorganisms viz. Alcaligenes fecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus epidermidis,
**Bacillus coagulans**, *Aspergillus bombycis* and *Saccharomyces bayanus* were procured from local hospitals.

**Isolation and Identification of Lactic Acid Bacteria**

The whey sample was collected in aseptic condition from local dairy plant in Barshi and processed within five hours for further studies. MRS broth and agar were used for enrichment and isolation of culture of Lactic Acid Bacteria. The sample was filtered through filter paper and 1ml sample was added into the 10ml MRS broth and incubated at 37°C for 24 hrs under Micro aeroophilic condition. After incubation, loopful of culture was spreaded on sterile MRS agar plate and all plates were incubated micro aeroically at 37°C for 24 hrs.

The well isolated colonies were selected randomly and transferred in MRS broth. They were streaked on MRS agar to check the purity of the isolates and then stored in MRS soft agar (0.5%) overlaid with glycerol at -20°C.

**Screening of bacteriocin producer**

The selected lactic acid bacterial isolates were cultured in MRS broth and incubated at same above mentioned conditions for 20 hrs. Aliquots of cultures were spotted on the sterile MRS agar plates and incubated micro aeroically at 37°C or 24 hrs. After incubation plates were overlaid with soft nutrient agar inoculated with culture of *Bacillus coagulans* and incubated at 37°C for 24 hrs under aerobic condition.

Isolate showing maximum zone of growth inhibition of *Bacillus coagulans* was selected and designated as WHL 3 and used for further studies.

**Bacteriocin production from selected isolate**

Bacteriocin production from selected isolate were carried out in three different medium viz. MRS broth, BSM (Bacteriocin Screening Medium) and M17 Medium. The selected culture was inoculated into above mentioned medium and incubated micro aeroically at 37°C for 24 - 48 hrs. During fermentation, aliquots of samples were removed for Growth curve and protein estimation analysis and results were recorded.

The medium showing maximum optical density in growth curve analysis and protein concentration was selected and used for further optimization studies.

**Optimization of some bacteriocin production conditions**

Some optimal conditions for cultivation of selected isolate were investigated to obtain efficient or maximum bacteriocin production. Optimization was done by changing one parameter and keeping other parameter constant. The composition of the medium showing maximum optical density and protein concentration in above step was varied as follows.

**Effect of Carbon and Nitrogen source**

Different Carbon and Nitrogen sources viz. Glucose, Fructose, Lactose, sucrose and Nitrogen source viz. Peptone, Triptone, Soyatone and Skimmed milk were used at 1% concentration.

**Concentration of yeast extract**

Yeast extract was applied as growth factor. The optimal concentration of yeast extract was also investigated. The concentration of Yeast extract viz. 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2% is added in medium containing optimum concentration of carbon and nitrogen source.

**Initial pH of bacteriocin production medium**

Initial pH of the optimized medium for maximum bacteriocin production was studied. The pH of medium was adjusted to 4 to 9 using 1N HCL and 1 N NaOH.

**Production temperature**

Suitable temperature for bacteriocin production was investigated. Various incubation temperatures such as 25, 30, 35, 40, 45, 50, 55 and 60°C were used for production studies.

**Production of bacteriocin using optimum conditions**

Bacteriocin production was performed in above optimized medium. 5ml of inoculum of WHL 3 isolate was inoculated in optimized medium and incubated micro aeroically at 37°C for 24 – 48 hrs. After fermentation broth was centrifuged at 12000rpm for 15 min. and supernatant was collected. pH of the supernatant was adjusted to 7.0 with 1N NaOH. Precipitation was done with Ammonium Sulphate at 40% and 70% saturation level at 4°C.

After precipitation, broth was centrifuged at 15000 rpm for 20 min., precipitate was collected and stored in 0.2M Sodium Phosphate buffer (pH 6.9) and labeled as Crude Bacteriocin Preparation (CBP).

**Extraction of Bacteriocin**

Chloroform – Methanol (2:1 v/v), was used for crude bacteriocin extraction. However produced precipitate at solvent – aqueous interphase was collected aseptically. Solvent was evaporated and precipitate was kept in buffer which was used for antimicrobial study.

**Antimicrobial activity of extracted bacteriocin**

Agar well diffusion and paper disc methods were used to study antimicrobial activity of extracted bacteriocin. Agar well diffusion technique was performed as 0.1ml culture of test microorganisms is spreaded on sterile nutrient agar and wells were prepared. 100µl of extracted bacteriocin preparation (CBP) was added wells and plates were aerobically incubated at 37°C for 24 hrs.

Paper disc assay was done as, paper discs were prepared from CBP and 0.1 ml culture of test microorganism was spreaded on nutrient agar plate and disc was placed. Plates were aerobically incubated at 37°C for 24 hrs. After incubation zone of growth inhibition of test microorganisms were recorded.
RESULTS

Isolation, Identification and Phylogenetic Analysis

Screening of bacteriocin producers were carried out by agar overlay method. In which the isolate showing maximum zone of growth inhibition of Bacillus coagulans was selected and used for production study (figure 1).

Lactic Acid Bacterial isolated designated as WHL 3 was selected as good candidate for bacteriocin production, based on its ability to form clear and large zone of growth inhibition of Bacillus coagulans. Molecular identification was carried out of this isolate based on 16s rRNA gene sequencing. The phylogenetic tree was constructed by using Neighbour joining method by Kimura 2 parameter with 1000 replicas in MEGA 4.0 (Figure 2).

According to sequence similarities and multiple alignments, the isolate WHL 3 was found to be in close relation to Lactobacillus rennanquilfy WHL 3.

Figure 1: Zone of growth inhibition of Bacillus coagulans by WHL 3 isolate

Figure 2: Phylogenetic tree of WHL 3 isolate

Figure 3: Estimation of protein in three different medium during fermentation by WHL 3 isolate
Production of bacteriocin before optimization

Bacteriocin production was carried out in three different medium viz MRS broth, BSM and M17 broth. The inoculum of selected WHL 3 isolate was added and incubated micro aerobically for 24 – 48 hrs during incubation, protein estimation and growth curve was studied at time intervals (fig.3& 4). Among them MRS is the best medium for bacteriocin production by Lactobacillus rennanquify WHL 3, as it shows maximum protein concentration. But maximum bacteriocin production was achieved in modified MRS medium.

Optimization of production conditions

To obtain maximum bacteriocin yield, production conditions including Carbon and Nitrogen source, Growth factors, Initial pH and Incubation temperature were investigated and results were showed in fig. (5 to 9). From that result it was observed that maximum bacteriocin production was achieved at 1% Glucose, 1% Peptone and 0.8% Yeast extract. And less bacteriocin production was observed in Sucrose, arabinose, Soyatone and skimmed milk containing medium.

Antimicrobial spectrum of Lactobacillus rennanquify WHL 3

Crude bacteriocin preparation obtained from isolated WHL 3 strain was tested for antimicrobial activity against test microorganisms viz. Alcaligenes fecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus epidermidis, Bacillus coagulans, Aspergillus bombycis and...
saccharomyces bayanus by agar well diffusion method. The antibiogram of bacteriocin from isolated WHL 3 strain against test microorganisms were shown in Table 1.

**Table 1**: Antibiogram of *Lactobacillus rennanquilfy* WHL 3 against test microorganisms

<table>
<thead>
<tr>
<th>Name of test Microorganisms</th>
<th>Zone diameter of growth inhibition of test Microorganisms in mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaligenes fecalis</td>
<td>20</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>24</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>23</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>20</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>18</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>26</td>
</tr>
<tr>
<td>Bacillus coagulans</td>
<td>21</td>
</tr>
<tr>
<td>Aspergillus bombycis</td>
<td>17</td>
</tr>
<tr>
<td>saccharomyces bayanus</td>
<td>16</td>
</tr>
</tbody>
</table>

Bacteriocin production is frequently regulated by pH, incubation temperature, Growth factors, carbon and nitrogen source. In specific cases, higher bacteriocin production levels have been recorded at suboptimal conditions. In our present investigation, we observed that isolate WHL 3 grows well and produce maximum protein in MRS medium, to obtain good yield of bacteriocin, the MRS medium was modified on the basis of results obtained in optimization studies. In optimization study we observe the maximum zone of growth inhibition of test microorganisms at 1% glucose, 1% peptone, 0.8% yeast extract, pH 6.0 and at temperature 40°C.

The above results correlates with results of Svetoslav (2005), in that he studied the production and optimization of bacteriocin from *Enterococcus faecium* ST 311LD. Our results were also similar to the results of Bing Han (2011), in that he studied optimization for bacteriocin production by *Lactobacillus plantarum* YJG. During present investigation, we studied the antimicrobial activity of bacteriocin produced from WHL 3 isolate, and found that it shows considerable zone of growth inhibition of test microorganisms listed above. Form that results it was clear that all test microorganisms used were sensitive to bacteriocin from WHL 3. This result shows similarity to the results of Asma Ansari (2012), in that they studied production and antimicrobial spectrum of bacteriocin from *Bacillus subtilis* KIBGE IB – 17.

**CONCLUSION**

Finally from this present study it was concluded that, the isolate *Lactobacillus rennanquilfy* WHL 3 shows good growth and bacteriocin production in above modified MRS medium. And from antimicrobial spectrum of WHL 3 it was concluded that all the test microorganisms used in study were sensitive to bacteriocin from WHL 3.

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