Optimization studies on production of bacteriocin from *Bacillus aryabhattai*

Pathade A.G.1 *and Bodhankar M.G.2*

1KIAS, Karad, Maharashtra, India
2BVDU, Pune, Maharashtra, India

(Received 9 July, 2023; Accepted 11 September, 2023)

**ABSTRACT**

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria and are active against other bacteria, either in the same species (Narrow spectrum) or across genera (broad spectrum). The optimization of medium and other conditions were studied with respect to carbon source, nitrogen source, pH, inoculum size, incubation period and incubation temperature using de Man Rogosa Sharpe (MRS) basal medium, and it was found that, *Bacillus aryabhattai* isolate showed maximum yields of bacteriocins in their respective optimized media as compared to basal medium. The medium and other conditions were optimized at 0.1-L flask level studies. The *Bacillus aryabhattai* is isolated from soil and identified by 16s rRNA gene sequencing and NCBI accession number is MG551546.1 and the phylogenetic tree is also prepared. The optimized conditions include 2% Glucose as carbon source, 1% NH4CL as nitrogen source, pH 6.0 in the MRS base medium, inoculum size of 6% at 108 Colony Forming Units (CFU)/ml of the suspensions, incubation period of 48 h and 30 °C temperature of incubation. These optimized conditions were used in the further work. The antagonistic potential against common food bacterial pathogens like pathogenic *Escherichia coli* and *Staphylococcus aureus* also carried out.

**Key words:** Bacteriocins, *Bacillus aryabhattai*, Production Conditions

**Introduction**

Bacteriocins are proteinaceous antibiotic like substances produced by bacteria which can inhibit the growth of similar or closely related bacteria (Calo-Mata et al., 2007). The bacteriocins are ecofriendly in the preservation of foods especially marine foods. The frozen fishes and their products are mainly export grade and in very little quantity they are used in local markets (MPEDA, 2014).

Globally, in general and in India particularly, huge quantities of foods are spoiled every year especially by microorganisms and cause incalculable losses to producers, handlers, stockists and industrialists. Many times packaged foods are unapproved and hence rejected as they harbor pathogenic and other microorganisms (Desriac, 2010).

In present work efforts have been taken for optimization studies on production of bacteriocin from *Bacillus aryabhattai isolate which* is obtained from soil and identified genetically. The study of their broad spectrum antagonistic potential against common food bacterial pathogens like pathogenic *Escherichia coli* and *Staphylococcus aureus* (Gálvez et al., 2007 and 2008) is also done.

**Materials and Methods**

**Screening and confirmation of bacteriocin nature** in the selected promising antagonistic (showing in-
hibitory activity) bacterial isolate *Bacillus aryabhattai* was done by using following tests as guideline: (Joshi et al., 2006; Balvir Kumar et al., 2011):

1. **Extraction of bacteriocins:** They are extractable in Chloroform: methanol (2:1) and salting out/precipitation at around 40% ammonium sulphate concentration.

2. **Increased protein content in the medium in case of bacteriocin producing isolates as compared to bacteriocin nonproducers.** (Protein estimation: Lowry method) Bacteriocins being proteinous in nature, their secretion in the medium cause increase in protein content of cultured medium.

3. **The loss of inhibitory activity (Inactivation) of the extract occurs upon trypsin treatment—as bacteriocin is proteinous in nature, it is destroyed by proteolytic enzyme “trypsin”.

4. **Inactivation (loss of Inhibition activity) at high temperatures-bacteriocin protein gets denatured.**

5. **The inhibitory activity may be because of secretion of acid / bacteriocin / toxic metabolites like H₂O₂ / antibiotics etc. in the medium by the bacterial isolates. The neutralization of medium extracts will remove possibility of acid secretion, treatment of neutralized medium extracts with catalase enzyme will remove possibility of toxic metabolite like H₂O₂ and loss of activity upon dilution will remove possibility of antibiotic.**

**The Optimization** for C, N sources in medium, pH, temperature, Inoculum size and incubation period were done with reference to (Dunder, 2006; Todorov et al., 2012 and Meera and Devi, 2012). The optimal conditions for bacteriocin production were investigated to obtain maximum bacteriocin. Optimization was done by changing one parameter and keeping other parameters constant. The MRS broth (100-ml) for isolate *Bacillus aryabhattai* was used throughout the studies.

**Optimization of Carbon source:** The 100 ml media in the 250 ml capacity flasks (without carbon source) were used for optimization studies. The carbon sources were selected on the basis of ease of availability, richness and the cost wise economically feasible. The 100 ml media in the 250 ml capacity flasks (without nitrogen source) were used for optimization studies. The carbon source which showed maximum bacteriocin activity (in terms of diameters of zones of inhibition) was recorded and considered as optimal carbon source.

**Optimization of Nitrogen source:** The nitrogen sources were selected on the basis of ease of availability, richness and the cost wise economically feasible. The 100 ml media in the 250 ml capacity flasks (without nitrogen source) were used for optimization studies. For optimization of nitrogen source, the nitrogen sources like peptone, tryptone, NH₄Cl, (NH₄)₂HPO₄·5H₂O and urea were added separately in 1% amounts the media as sole nitrogen sources and selected *Bacillus aryabhattai* was grown separately in static condition in the incubator in triplicate sets at following conditions: Temperature of incubation- 30°C, pH of the medium- 6.5 Incubation period: 48 h. Inoculum size- 5% at 10⁸ CFU/ml suspensions of isolate. After incubation, the cell free extracts (membrane filtered) were catalase treated as described above and then subjected for estimation of bacteriocin activity using MRS agar paper disc diffusion method (Schillinger et al., 1991; Cruickshank et al., 1985) and *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The carbon source which showed maximum bacteriocin activity (in terms of diameters of zones of inhibition) was recorded and considered as optimal carbon source.

**Optimization of pH of the medium:** Influence of initial pH on bacteriocin production was also investigated. The 100 ml media in the 250 ml capacity flasks were used for optimization studies. The pH of media (with optimized carbon and nitrogen sources) was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, separately using 1N HCl and 1N NaOH and selected *Bacillus aryabhattai* isolate was grown in static condition in the incubator in triplicate sets at following conditions: Carbon source- 2% glucose (optimized), Nitrogen source- 1% NH₄Cl (optimized), Incubation period- 48 h, Inoculum size- 5% at 10⁸ CFU/ml of suspension of isolate, Temperature of incubation- 30°C After incubation, the cell free extracts (membrane filtered) were catalase treated as...
described above and subjected for the estimation of bacteriocin activity using MRS agar paper disc diffusion method, *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The pH with which maximum bacteriocin activity (in terms of diameters of zones of inhibition) recorded, was considered as optimal pH of medium for bacteriocin production.

**Optimization of incubation temperature of medium:** Incubation temperature is one of the important factors for achieving maximum bacteriocin production. Suitable temperature for bacteriocin production was investigated. The bacterial isolate is isolated at ambient temperature (around 28-31°C) and hence temperatures around it were selected for the study. The 100 ml media in the 250 ml capacity flasks were used for optimization studies. After inoculations with selected *Bacillus aryabhattai* isolate separately, the flasks were incubated in static conditions in the incubator separately in triplicate sets at temperatures of 25, 30, 35, 40, and 45°C and with following conditions: Carbon source- 2% glucose (optimized), Nitrogen source- 1% NH4Cl (optimized), pH of the medium- 6.0 (optimized), Inoculum size- 5% at 10^8 CFU/ml suspension of isolate, 48-h incubation period. After incubation, the cell free extracts (membrane filtered) were catalase treated as described above and subjected for estimation of bacteriocin activity using MRS agar paper disc diffusion method, *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The incubation temperature, with which maximum bacteriocin activity (in terms of diameters of zones of inhibition) recorded, was considered as optimal incubation temperature for bacteriocin production by isolate.

**Optimization of incubation period:** Incubation period is one of the important factors for achieving maximum bacteriocin production. Suitable incubation period for bacteriocin production was investigated. The 100 ml media in the 250 ml capacity flasks were used for the optimization studies. After inoculations with selected *Bacillus aryabhattai* isolate separately, the flasks were incubated in static conditions in the incubator separately in triplicate sets at incubation periods of 12, 24, 36, 48, 60 and 72-h and with following conditions: Carbon source- 2% glucose (optimized), Nitrogen source- 1% NH4Cl (optimized), pH of medium- 6.0 (optimized), Incubation period- 48 h (optimized) and Incubation temperature 30°C (optimized). In the most of fermentations 5-10% of inoculum is used hence inoculum doses selected were in the range from 2, 4—10%. After incubation, the cell free extracts (membrane filtered) were catalase treated as described above and subjected for estimation of bacteriocin activity using MRS agar paper disc diffusion method and *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The incubation period with which maximum bacteriocin activity (in terms of diameters of zones of inhibition) recorded, was considered as optimal incubation period for bacteriocin production by isolate.

**Optimization of inoculum size (dose):** The inoculum size is one of the important factors for achieving maximum bacteriocin production. Suitable inoculum size for bacteriocin production was investigated. The 100 ml media in the 250 ml capacity flasks were used for the optimization studies. The inoculum sizes/ doses selected were 2, 4, 6, 8 and 10% Log CFU/ml. After inoculation with selected *Bacillus aryabhattai* isolate separately, the flasks were incubated in the static conditions in the shaker incubator separately in triplicate sets with following conditions: Carbon source- 2% glucose (optimized), Nitrogen source- 1% NH4Cl (optimized), pH – 6.0 (optimized), Incubation period- 48 h (optimized) and Incubation temperature 30°C (optimized). In the most of fermentations 5-10% of inoculum is used hence inoculum doses selected were in the range from 2, 4—10%. After incubation, the cell free extracts (membrane filtered) were catalase treated as described above and subjected for estimation of bacteriocin activity using MRS agar paper disc diffusion method and *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The inoculum size, with which maximum bacteriocin activity (in terms of diameters of zones of inhibition) recorded, was considered as inoculum size optimal for bacteriocin production by isolate.
Results and Discussion

*Bacillus aryabhattai*: Phylogenetic tree

**Bacteriocins production studies**

Screening and confirmation of bacteriocin nature in the selected promising antagonistic (showing inhibitory activity) bacterial isolate, *Bacillus aryabhattai*. Bacteriocin confirmation was done by using following tests as guideline: (Joshi et al., 2006, Balvir Kumar et al., 2011) and found that they were extractable in Chloroform: methanol (2:1) and salted out/precipitation at around 40% ammonium sulphate concentration, increased protein content of medium, as Bacteriocins being proteinous in nature, their secretion in the medium caused increase in protein content of cultured medium, the loss of inhibitory activity (Inactivation) of the extract occurred upon trypsin treatment—as bacteriocin is proteinous in nature, it is destroyed by proteolytic enzyme “trypsin”, inactivated (loss of Inhibition activity) at high temperatures-bacteriocin protein, the inhibitory activity may be because of secretion of acid / bacteriocin / toxic metabolites like H2O2 / antibiotics etc. in the medium by the bacterial isolate. The culture extract was neutralized, treated with catalase enzyme and used upon excessive which still retained inhibitory activity indicating bacteriocin nature of culture extract.

**Media optimization Studies**

**Optimization of Carbon source**

The results of optimization of carbon sources are presented in Table 1. It is evident from the results that with *E.coli* and *S. aureus* as test pathogens, maximum 25 and 24 mm and minimum 14 and 13 mm, diameters of zones of inhibition were obtained with glucose and arabinose as carbon sources, respectively. Thus it is clear that the 2% glucose was the optimum carbon source followed by lactose and sucrose, and then arabinose.

**Optimization of Nitrogen source**

The results of optimization of nitrogen sources are presented in Table 2. It is evident from the results that with *E.coli* and *S. aureus* as test pathogens, maximum 27 mm and 27 mm diameters of zones of inhibitions were obtained, respectively, in case of NH4Cl as nitrogen source, It is found that the nitrogen sources of next choice are urea and (NH4)2HPO4.5H2O followed by peptone and tryptone. Thus it is clear that the 1% NH4Cl was the optimum nitrogen source.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Nitrogen source 1%</th>
<th>Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <em>Bacillus aryabhattai</em> against test pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>1</td>
<td>Peptone</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Tryptone</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>NH4Cl</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>(NH4)2HPO4.5H2O</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Urea</td>
<td>26</td>
</tr>
</tbody>
</table>

**Optimization of pH of medium**

The results of optimization of pH are presented in Table 3. It is evident from the results that with *E.coli* and *S. aureus* as test pathogens, maximum 27 mm and 27 mm diameters of zones of inhibitions were obtained, respectively, in case of pH 6.8 as nitrogen source, It is found that the pH sources of next choice are pH 6.0 and 5.5 followed by pH 5.0 and 4.5. Thus it is clear that the pH 6.8 was the optimum pH source.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>pH of the medium</th>
<th>Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <em>Bacillus aryabhattai</em> against test pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
and *S. aureus* as test pathogens, maximum 28 mm and 27 mm diameters of zones of inhibitions, respectively, were obtained, in case of pH 6.0 of the medium.

Thus it is clear that the pH 6.0 was the optimum for the production of bacteriocins under study.

### Table 4. Optimization of incubation temperature of medium

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Temp. of the medium (°C)</th>
<th>Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <em>Bacillus aryabhattai</em> against test pathogens:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em> <em>Bacillus aryabhattai</em> <em>S. aureus</em> <em>Bacillus aryabhattai</em></td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>20                                                                  22</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>30                                                                  28</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>27                                                                  25</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>17                                                                  14</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>9                                                                   7</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>0.0                                                                 0.0</td>
</tr>
</tbody>
</table>

### Optimization of incubation temperature

The results of optimization of incubation temperature are presented in Table 4. It is evident from the results that with *E. coli* and *S. aureus* as test pathogens, maximum 30 and 28 mm diameters of zones of inhibitions were obtained, respectively, in case of 30 °C incubation temperature. Thus it is clear that the 30 °C incubation temperature was the optimum.

### Table 5. Optimization of incubation period

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Incubation period (h)</th>
<th>Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <em>Bacillus aryabhattai</em> against test pathogens:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em> <em>Bacillus aryabhattai</em> <em>S. aureus</em> <em>Bacillus aryabhattai</em></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>10                                                                  8</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>17                                                                  14</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>24                                                                  22</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>31                                                                  28</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>28                                                                  22</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>20                                                                  6</td>
</tr>
</tbody>
</table>

### Optimization of inoculum size/Dose

The results of optimization of inoculum size/dose are presented in Table 6. It is evident from the results that with *E. coli* and *S. aureus* as test pathogens, maximum 32 mm and 28 mm inhibition zone diameters were obtained in case of 6% inoculum size/dose. Thus it is clear that the 6% inoculum size/dose was the optimum for bacteriocin production under study.

### Optimized conditions

Optimized conditions for production of bacteriocins using isolate *Bacillus aryabhattai*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Condition</th>
<th>Optimized value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH of the medium</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>Temperature of incubation (°C)</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Carbon source</td>
<td>2% glucose</td>
</tr>
<tr>
<td>4</td>
<td>Nitrogen source</td>
<td>1% NH₄Cl</td>
</tr>
<tr>
<td>5</td>
<td>Incubation period (h)</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>Inoculum size</td>
<td>6% at 10⁹ CFU/ml of the suspension</td>
</tr>
</tbody>
</table>
Production studies using optimized set of conditions

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Bacteriocin of No. Isolate type</th>
<th>Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) isolate Bacillus aryabhattai against test pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus aryabhattai</td>
<td>E. coli: 37, S. aureus: 35</td>
</tr>
</tbody>
</table>

There was increase in yield of bacteriocin of Bacillus aryabhattai isolate upon using set of all optimized conditions. In the optimization process the maximum yield (in terms of diameters of zones of inhibition in the assay with test pathogens) was 32 mm diameter of zone of inhibition in the assay with test pathogens but after use of all optimized conditions it was increased to 37 mm for E.coli and 35 mm for S.aureus.

Conclusion

1. The Bacillus aryabhattai bacteriocin exhibited maximum inhibitory activity against test bacterial pathogens.
2. The 2% Glucose as carbon source, 1% NH₄CL as nitrogen source, pH 6.5in the MRS base medium, inoculum size of 6 % at 10⁶ CFU/mL of the suspension and 30°C temperature of incubation were found to be optimum conditions for production of bacteriocins using Bacillus aryabhattai.

References


