

# Optimization studies on production of bacteriocin from *Bacillus aryabhatai*

Pathade A.G.<sup>1</sup>\* and Bodhankar M.G.<sup>2</sup>

<sup>1</sup>KIAS, Karad, Maharashtra, India

<sup>2</sup>BVDU, 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 aryabhatai* 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 aryabhatai* 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% NH<sub>4</sub>CL as nitrogen source, pH 6.0 in the MRS base medium, inoculum size of 6 % at 10<sup>8</sup> 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 aryabhatai*, 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, stockist 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 aryabhatai* 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 aryabhatai* 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 at high temperatures.
5. The inhibitory activity may be because of secretion of acid / bacteriocin / toxic metabolites like  $H_2O_2$  / 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  $H_2O_2$  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 aryabhatai* 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 and economic feasibility. For optimization of carbon source the carbon sources (2%) like arabinose (as pentose sugar), glucose (as simple monosaccharide), lactose and sucrose (as common disaccharides), were added in the separate medium as sole carbon sources and selected *Bacillus aryabhatai* isolate was grown in static condition in the incubator in triplicate sets at following conditions: Tempera-

ture of incubation- 30 °C, pH of the medium- 6.5 Incubation period: 48 h. Inoculum size- 5% at  $10^8$  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 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,  $NH_4Cl$ ,  $(NH_4) 2HPO_4 \cdot 5H_2O$  and urea were added separately in 1% amounts the media as sole nitrogen sources and selected *Bacillus aryabhatai* 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- 48h, Carbon source (Glucose) - 2% (optimized), Inoculum size- 5% at  $10^8$  CFU/ml suspension of isolate. 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 nitrogen source, with which maximum bacteriocin activity (in terms of diameters of zones of inhibition) recorded, was considered as optimal nitrogen 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 aryabhatai* isolate was grown separately in static condition in the incubator in triplicate sets at following conditions: Carbon source- 2% glucose (optimized), Nitrogen source- 1%  $NH_4Cl$  (optimized), Incubation period- 48 h, Inoculum size- 5% at  $10^8$  CFU/ml of suspension of isolate, Temperature of incubation- 30 °C After incubation, the cell free extracts (membrane filtered) were catalase treated as

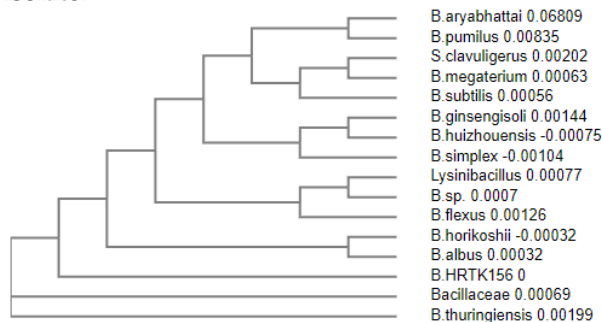
described above and subjected for the estimation of bacteriocin activity using MRS a gar paper disc diffusion method, *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The pH with which maximum bacteriocin activity (in terms of diameters of zones of inhibition) recorded, was considered 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 aryabhatai* 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% NH<sub>4</sub>Cl (optimized), pH of the medium- 6.0 (optimized), Inoculum size- 5% at 10<sup>8</sup> 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 the 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 aryabhatai* isolate separately, the flasks were incubated in static condition 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% NH<sub>4</sub>Cl (optimized), pH of medium – 6.0 (optimized), Incubation temperature- 30 °C (optimized), Inoculum size- 5% at 10<sup>8</sup> CFU/ml suspension of isolate Incubation

temperature- 30°C (optimized). The bacteriocins are primary metabolic products of bacteria; hence their production starts in the incubation periods (10-12-h) to late Log phases of growth. Therefore, incubation period range of 12-72-h was used. After incubations, 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 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 aryabhatai* 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% NH<sub>4</sub>Cl (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 aryabhatai*: Phylogenetic tree

#### Bacteriocins production studies

**Screening and confirmation of bacteriocin nature** in the selected promising antagonistic (showing inhibitory activity) bacterial isolate, *Bacillus aryabhatai*. 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  $H_2O_2$  / 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,

**Table 1.** Optimization of Carbon source

Sr. No.	Carbon source (2%)	Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <i>Bacillus aryabhatai</i> against test pathogens	
		<i>E. coli</i>	<i>S. aureus</i>
		<i>Bacillus aryabhatai</i>	<i>Bacillus aryabhatai</i>
1	Glucose	25	24
2	Lactose	22	18
3	Sucrose	22	20
4	Arabinose	14	13

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  $NH_4Cl$  as nitrogen source, It is found that the nitrogen sources of next choice are urea and  $(NH_4)_2HPO_4 \cdot 5H_2O$  followed by peptone and tryptone. Thus it is clear that the 1%  $NH_4Cl$  was the optimum nitrogen source.

**Table 2.** Optimization of Nitrogen source

Sr. No.	Nitrogen source 1%	Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <i>Bacillus aryabhatai</i> against test pathogens	
		<i>E. coli</i>	<i>S. aureus</i>
		<i>Bacillus aryabhatai</i>	<i>Bacillus aryabhatai</i>
1	Peptone	23	20
2	Tryptone	23	21
3	$NH_4Cl$	27	27
4	$(NH_4)_2HPO_4 \cdot 5H_2O$	22	23
5	Urea	26	23

##### 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*

**Table 3.** Optimization of pH of medium

Sr. No.	pH of the medium	Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <i>Bacillus aryabhatai</i> against test pathogens	
		<i>E.coli</i>	<i>S.aureus</i>
		<i>Bacillus aryabhatai</i>	<i>Bacillus aryabhatai</i>
1	3	0.0	0.0
2	4	0.0	0.0
3	5	12	10
4	6	28	27
5	7	27	25
6	8	7	9

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

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

#### 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

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

#### Optimization of incubation period

The results of optimization of incubation period are presented in Table 5. It is evident from the results that with *E.coli* and *S.aureus* as test pathogens, maximum 31 and 28 mm, inhibition zone diameters were obtained, respectively, in case of 48h incubation period. Thus it is clear that the 48h incubation period was the optimum for bacteriocin production under study.

**Table 6.** Optimization of inoculum size/Dose

Sr. No.	Inoculum size (%)	Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of the isolate <i>Bacillus aryabhatai</i> against test pathogen	
		<i>E.coli</i> <i>Bacillus aryabhatai</i>	<i>S.aureus</i> <i>Bacillus aryabhatai</i>
1	2.0	13	15
2	4.0	21	23
3	<b>6.0</b>	<b>32</b>	<b>28</b>
4	8.0	31	26
5	10.0	25	22

#### 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 aryabhatai*

Sr. No.	Condition	Optimized value
1	pH of the medium	6.0
2	Temperature of incubation (°C)	30
3	Carbon source	2% glucose
4	Nitrogen source	1% NH <sub>4</sub> Cl
5	Incubation period (h)	48
6	Inoculum size	6 % at 10 <sup>8</sup> CFU/ml of the suspension

## Production studies using optimized set of conditions

Sr. No.	Bacteriocin of Isolate type	Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) isolate <i>Bacillus aryabhatai</i> against test pathogen	
		<i>E. coli</i>	<i>S. aureus</i>
1	<i>Bacillus aryabhatai</i>	37	35

There was increase in yield of bacteriocin of *Bacillus aryabhatai* 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 aryabhatai* bacteriocin exhibited maximum inhibitory activity against test bacterial pathogens.
2. The 2% Glucose as carbon source, 1% NH<sub>4</sub>CL as nitrogen source, pH 6.5 in the MRS base medium, inoculum size of 6 % at 10<sup>8</sup> CFU/mL of the suspension and 30°C temperature of incubation were found to be optimum conditions for production of bacteriocins using *Bacillus aryabhatai*.

### References

Calo-Mata, P., Arlindo, S., Boehme, K., Miguel, T., Pascoal, A. and Barros V.J. 2007. Current Applications and Future Trends of Lactic Acid Bacteria and their Bacteriocins for the Biopreservation of Aquatic Food Products. *Food and Bioprocess Technol.* 1(1): 43-63. ISSN 1935-5130.

- Cruickshank, R., Duguid J. P. and Marmion, B.P. and Swain, R.H.A. 1985. *Medical Microbiology*, Vol II, 12th Edn., Churchill Livingstone, London.
- Balvir, k., Balgir, P. P., Kaur, B. and Garg, N. 2011. Cloning and expression of bacteriocins of *Pediococcus spp.*: A Review. *Archives of Clin. Microbiol.* 2(3), doi: 10:3823/231.
- Joshi, V.K., Sharma, S. and Rana, N.S. 2006. Bacteriocin from lactic acid fermented vegetables. *Food Technol. Biotechnol.* 44 (3): 435-439.
- Desriac, F., Defer, D., Bourgougnon, N., Brillet, B., Le Chevalier, P. and Fleury, Y. 2010. Bacteriocin as weapons in the marine animal-associated bacteria warfare: Inventory and potential applications as an aquaculture probiotic. *Marine Drugs.* 8(4): 1153-1177, ISSN 1660-3397.
- Gálvez, A., Abriouel, H., Lucas, H., López, R. and Omar, N.B. 2007. Bacteriocin-based strategies for food biopreservation. *International J. of Food Microbiol.* 120: 51-70.
- Galvez, A., Lopez, R. L., Abriouel, H., Valdivia, E. and Omar, N.B. 2008. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. *Crit. Rev. in Biotechnol.* 28(2): 125-152, ISSN 1549-7801.
- Todorov, S.D., Oliveira, R.P.S. and Vaz-Velho, M. 2012. Media Optimisation of bacteriocin ST 22ch production by *Lactobacillus sakei* ST 22ch isolated from Salpicão, a traditional meat product from Portugal. *Chem. Engg. Trans.* 27: 283-288.
- Meera, N.S. and Charitha D.M. 2012. Partial characterization and optimization of parameters for bacteriocin production by probiotic lactic acid bacteria. *J. Microbiol. Biotechnol. Res.* 2(2): 357-365.
- Dunder, H. 2006. *Characterization and purification of a bacteriocin produced by Leuconostocmesenteroides sub sp. cremoris.* In: Ph.D. thesis, Graduate school of natural and applied sciences, Middle East Technical University. 1-218.
- Marine Products Export Development Authority (MPEDA) Manual, 2014. Cochin, India.
- Schillinger, U., Kaya, M. and Lucke, F.K. 1991. Behavior of *Listeria monocytogenes* in meat and its control by a bacteriocin producing strain of *Lactobacillus sakei*. *J. Appl. Bacteriol.* 70: 473-478.