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Optimization of production conditions for bacteriocins of Lactic Acid Bacterial (LAB) isolates

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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 16 out of 20 LAB isolates showed zones of growth inhibition against one or more of the test pathogens. The two promising isolates were identified as *Pediococcus pentosaceus* strain –I and *Pediococcus pentosaceus* strain –II *by 16s-rRNA* gene sequencing method. These two isolates were subjected to optimization studies. 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 deManRogosa Sharpe (MRS) basal medium, and it was found that, bothLABisolates showed maximum yields of bacteriocins in their respective optimized mediaas compared to basal medium. The medium and other conditions were optimized at 0.1-L flask studies. The optimized conditions include 2% Glucose as carbon source, 1% NH_4CL as nitrogen source, pH 6.5 in the MRS base medium, inoculum size of 6 % at 10^8Colony Forming Units (CFU)/ml of the suspensions, incubation period of 48-h and 30 ^oC temperature of incubation. These optimized conditions were used in the further work.

Key words : *Bacteriocins, LAB, Production Conditions*

Introduction

Bacteriocins are proteinaceous antibiotic like substances produced by bacteria which can inhibitthe growth of similar or closely related bacteria (Calo-Mata *et al.,* 2007).

In India it is must to use unconventional methods for preservation of foods and their global acceptability. This can be achieved by using a method like use of bacteriocins which have negligible environmental issues and are being used by some countries like USA, Japan and UK (Desriac *et al.*, 2010). Bacteriocins have numerous qualities that make them attractive as alternatives to antibiotics. They have been shown to be non-toxic to eukaryotic cells and are GRAS (Generally Regarded As Safe), making them a safe alternative to traditional antimicrobials. It has also been shown that purified bacteriocins do not affect the sensory qualities of food.

James *et al.,* 1991 reported that among the microbial flora identified Lactic Acid Bacteria (LAB) remain the category that offers the higher potential for application for bacteriocin production. Bacteriocins are found in almost every bacterial species, and within the species, strains about hundreds of different kinds of bacteriocins are produced.

Todorov *et al.,* 2012 reported maximum antimicrobial activity in medium containing Tryptone and

meat extract.

The optimization of production conditions for bacteriocin is important for achieving target of more production of bacteriocins for use in food preservation.

Materials and Methods

LAB isolates: Two LAB isolates, i.e. *Pediococcus pentosaceus* strain –I and *Pediococcus pentosaceus* strain –II which were promising isolates producing bacteriocin.

Optimization of media and fermentation conditions

The optimal conditions for bacteriocin production were investigated to obtain maximum bacteriocin. Optimization was done by changing one parameter and keeping other parameter constant with respect to carbon, nitrogen sources, pH of medium, incubation temperature and period and inoculums size of isolates. The MRS broth was used for isolates **L15**: *Pediococcus pentosaceus* strain –I and **L19**: *Pediococcus pentosaceus* strain –II. After incubation, the cell free extracts (membrane filtered) were treated with catalase and then subjected for estimation of bacteriocin activity using MRS agar paper disc diffusion method (Cruickshank *et al.*, 1985 and Shillinger *et al.*, 1991) and *Staphylococcus aureus* and *E.coli* as bacterial test pathogens. The 100 ml media in the 250 ml capacity flasks were used for the optimization studies. The carbon sources were selected on the basis of ease of availability and economic feasibility.

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 two promising isolates were added. Separately 5% at 10^8 CFU/ml suspensions of each isolates and incubated in static condition in the incubator in triplicate sets. The carbon source which showed maximum bacteriocin activity (in terms of diameters of zones of inhibition) was 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 economic feasibility. For optimization of nitrogen source, the nitrogen sources like peptone, tryptone, NH_4Cl, $(NH_4)_2HPO_4.5H_2O$ and urea were added separately in 1% amounts the media as sole nitrogen sources. 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 medium

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. 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

Incubation temperature is one of the important factors for achieving maximum bacteriocin production. The bacterial isolates are isolated at ambient temperature (around 28-31°C) and hence temperatures around it were selected for study. The flasks were incubated in static conditions in the incubator separately in triplicate sets at temperatures of 25, 30, 35, 40, and 45 °C. The incubation temperature, with which maximum bacteriocin was considered as optimal incubation temperature for bacteriocin production by isolates.

Optimization of incubation period

Incubation period is one of the important factors for achieving maximum bacteriocin production. The flasks were incubated in static condition in the shaking incubator separately in triplicate sets at incubation periods of 12, 24, 36, 48, 60 and 72-h. 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. 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 isolates.

Optimization of inoculum size

Inoculum size is one of the important factors for achieving maximum bacteriocin production. The inoculum sizes/ doses selected were 2, 4, 6, 8 and 10 % Log CFU/mL. 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 isolates.

Bacteriocin production studies using optimized set of conditions (250ml level- flask studies)

The 100 ml media (MRS broth) were used for these production studies in the 250 ml capacity flasks. After inoculations (6 ml suspensions of each of selected three promising isolates at 10^8 CFU/ml] in the separate sets of batch fermentations, the batches were run with the set of optimized conditions (Table 1) and the flasks were incubated in incubator in triplicate sets. After incubations, the cell free extracts (membrane filtered) were catalase treated 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 average bacteriocin activity of each of the three promising isolates was recorded in terms of diameters of zones of inhibition.

Results and Discussion

Optimization of media and fermentation conditions

The optimized conditions regarding media constituents and other conditions are shown in Table 1.

Optimized conditions

The present results also correlate with the findings reported by Oh *et al.,* 2000 where they observed maximum bacteriocin yield in MRS medium by potential probiotic culture *Lactobacillus acidophilus 30SC*. Cheigh *et al.* (2002) reported that the maximum bacteriocin was produced at stationery phase where maximum biomass was formed. As per findings of Aasen *et al.,* 2003 rate of bacteriocin production increases with increasing concentration of yeast extract up to certain extent. Coeuret *et al.,* (2003) and cultured lactic acid bacteria in BSM and M17 media. They observed maximum protein concentration and antimicrobial activity. Todorov *et al*., (2012) reported maximum antimicrobial activity in medium containing Tryptone and meat extract. A number of reports published earlier by many researchers also observed maximum antimicrobial activity in above mentioned fermentation conditions. Malini and Savitha, 2012 at 37 ^{0}C incubation temperature, pH 7.0 of medium and at 0.2% NaCl obtained a maximum bacteriocin production. They also suggested that, Glucose, Fructose, Mannitol, Lactose and Sucrose are the best carbon source for bacteriocin production from LAB. Meera and Charitha, (2012) produced bacteriocin in medium containing 2g/100 ml glucose, 2g /100l l yeast extract, g /100 ml NaCl, at 6.0 pH and incubated at 30 ^{0}C temperature and in that study they observed antimicrobial activity ≈ 15800 AU/ml in optimized medium, while ≈13000 AU/ml antimicrobial activity in unoptimized medium. Deshmukh, in 2015 reported correlation between amounts of bacteriocin produced with that of extent of biomass accumulated.

Production studies using optimized set of conditions (Table 2 and Fig. 1): (at 250 ml level flask studies)

When the production studies using optimized set of conditions were done, it was observed that the bacteriocin activity was increased as compared to the activity obtained during the process of optimization, indicating the conditions used in production were appropriately optimized.

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Table 1. Optimized conditions for production of bacteriocins using isolates **L15**: *Pediococcus pentosaceus* strain –I and **L19**: *Pediococcus pentosaceus* strain –II:

Sr.No.	Condition	Optimized value
1	pH of the medium	6.0
2	Temperature of incubation (^{0}C)	30
3	Carbon source	2% glucose
4	Nitrogen source	1% NH_4Cl
5	Incubation period (h)	48
6	Inoculum size	6 % at 10^8 CFU/ml of the suspension

Table 2. Production studies using optimized set of conditions

Sr. No.	Bacteriocin of Isolate type	Diameter of zone of inhibition (mm) shown by bacteriocins (filter sterilized) of three isolates (L-15, L-19 and ISO6 RNFA) against test pathogen	
		E.coli	*S.aureus*
1	L_{15}	36	34
2	L_{19}	30	31
3	ISO6 RNFA	37	35

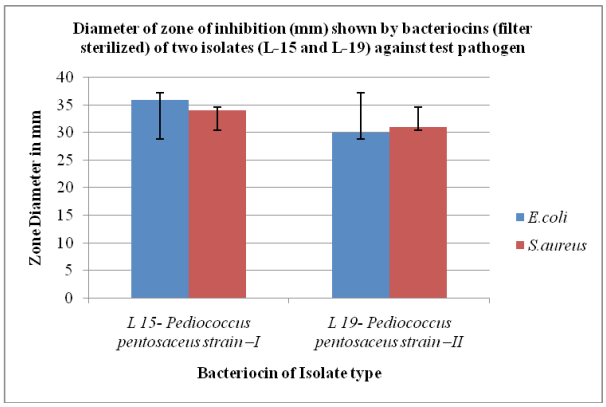

Fig. 1. Production studies (250 ml flask studies) using optimized set of conditions

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