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EC MICROBIOLOGY
Research Article

Optimization of Media and Culture Conditions for Improved Production of Bacteriocin by Using Conventional One-Factor-At-A-Time (OFAT) Method

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Received: June 04, 2018; **Published:** March 25, 2019

Abstract

In this study, bacteria were isolated from various acidic food samples like curd, milk, paneer, cheese, guava, papaya, mosambi etc. Only one strain isolated from perishable papaya was selected on MRS (deMan, Rogosa, Sharpe) agar and isolation, characterization and purification of its bacteriocin was achieved. For making this bacteriocin industrially viable, its culture conditions along with different media components were optimized using conventional One-Factor-At-A-Time (OFAT) method. It was found that lactose 4%, yeast extract 1.5%, di-potassium hydrogen phosphate 0.4% and tween 80 0.2% showed maximum bacteriocin production. 37°C temperature and 6.8 pH were found to be most suitable for the maximum bacteriocin production.

Keywords: *One-Factor-At-A-Time (OFAT); Bacteriocin*

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that inhibit the growth of similar or closely related bacterial strains [1] and have received awareness in recent years due to their possible therapeutic applications in treating bacteria including multiple drug resistant bacteria [2-4]. Bacteriocins produced by lactic acid bacteria (LAB) is receiving increased importance as food biopreservative [5] especially due to their low toxicity and natural biodegradability by digestive enzymes [6]. They are a favourable tool for improvement in food safety and may replace or reduce the use of harmful chemical additives (Juneja., *et al.* 2012). Many bacteriocins produced by LAB are already known and they vary in their spectrum of antimicrobial activity (narrow or broad), molecular masses, modes of action, and genetic and biochemical properties [7]. Commercial formulations containing bacteriocins such as Nisaplin (nisin) and ALTA (pediocin PA-1) have been added to food to enhance shelf life and increase food safety [8]. However, some bacteria are recently found showing resistance to Nisin [9] or produce proteolytic substances that inhibit its action [10]. In this context, searching for other potential LAB bacteriocins has become crucial to overcome the shortcomings of the classic antimicrobials used in food and pharmaceutical industries.

Production of bacteriocins by LAB depends on bacterial growth and maximum activity is usually synchronous with maximum cell growth [11]. However, environmental factors like temperature, pH and media composition can affect the extent of bacteriocin production [12]. MRS (deMan, Rogosa, Sharpe) broth is the most commonly used growth medium for the cultivation of LAB [13]. Certain components, such as carbohydrates, salts, surfactants or oxygen tension reducing agents added to the MRS broth can interfere with bacteriocin production [14,15]. These factors need to be studied well to achieve optimized bacteriocin production for industrial applications.

In this context, the present investigation was undertaken to optimize the bacteriocin production by *Lactococcus lactis* JC10 with varying culture conditions and media components for its further application in the field of biological food preservation. Several reports

have shown that complex media and well controlled physical factors such as temperature and pH are required for optimal bacteriocin production. Bacteriocin production can be influenced by media composition and growth phase of microorganism [16]. The production of bacteriocin is usually studied on complex rich media and the most recently evaluated parameters are the concentration of carbon source, complex nitrogen source and tween 80. Hence the present attempt has been undertaken to investigate the influence of various culture conditions and media components on bacteriocin production by *Lactococcus lactis* JC10 [17].

Materials and Methods

Maximization of bacteriocin production

Determination of bacteriocin production at different culture conditions

The effects of different temperature and initial pH on the bacteriocin production were tested. The effects of different temperatures and initial pH on the bacteriocin production were tested. MRS broth (5 mL) was inoculated with the isolated LAB strain and incubated at different temperatures such as 10°C, 27°C, 37°C, 42°C, 45°C and 65°C to study the effect of different temperatures on the bacteriocin production. The effect of initial medium pH on bacteriocin production was determined by adjusting the MRS broth to different pH levels of 4.5, 5.5, 6.8, 7.5 and 8.5 respectively by using 1N NaOH and 1N HCl in the culture medium. Each tube was inoculated with 1.0% v/v of 24 h-old culture of the isolate and incubated at 37°C 6 hrs in static condition.

Influence of incubation time on bacteriocin production

Incubation time plays a vital role in bacteriocin production. LAB isolate was grown in MRS medium. Flask containing 50 ml of sterilized MRS broth was inoculated with 1% (v/v) bacteriocin-producing LAB strain and incubated at 37°C and allowed to grow for 6 hours at static condition. At different time interval of 30 minutes, 2 ml of culture was taken out and centrifuged at 10000 rpm for 10 minutes in a cooling centrifuge (4°C). 5 µl of CFS was spotted on lawn of indicator strain (*Lactobacillus plantarum* 2083) to determine maximum production of antimicrobial compound with relation to microbial growth. The antibacterial activity was expressed as AU/mL and defined as

AU/mL= Diameter of Zone of Inhibition (mm) ×1000/Volume taken in well (**µ**L)

Here the volume taken was 5 **µ**L.

So, AU/mL simplifies to= Diameter of Zone of Inhibition (mm) × 200 (Barbosa M.S et al. 2013)

Protein content in culture broth was evaluated by RC DC protein assay system (Bio-Rad).

Optimization of media components

The production media used in this study is MRS (DeMan, Rogosa, Sharpe) medium (HiMedia) and its composition in g/L is as follows: Proteose peptone: 10.0, Beef extract: 10.0, Yeast extract: 5.0, Dextrose: 20.0, Tween 80: 1.0, Ammonium citrate: 20.0, Sodium citrate: 5.0, Magnesium sulphate: 0.1, Manganese sulphate: 0.05, Dipotassium Hydrogen Phosphate: 2.0 at a final pH of 6.5.

MRS (Himedia) contained three nitrogen sources: protease peptone, yeast extract, beef extract, tryptone. In this study it was varied separately or in several different combinations. Metal ions source (salt sources like sodium acetate, tri ammonium citrate, di ammonium hydrogen phosphate) and carbohydrate sources (glucose, maltose, mannitol, galactose, sucrose, lactose etc.) were varied in different concentrations in basal medium one at a time. All other medium ingredients in basal medium were kept constant when one component was varied.

To achieve maximum bacteriocin production by *Lactococcus lactis* JC10, the various media components like carbon sources (glucose, maltose, galactose, mannitol, mannose, sucrose, lactose at concentrations ranging from 0.5% to 4%), Nitrogen sources (Proteose peptone, beef extract, yeast extract at varying concentrations ranging from 0.5% to 2%) were substituted in the production medium. Similarly different surfactants (tween 20, tween 40, tween 60, tween 80, SDS, Triton-X100 at different concentrations ranging from 0.05% t0 0.2%) were supplemented in the production medium. Appropriate control of MRS medium was also maintained in each case.

Then all the flasks were inoculated aseptically with 1% v/v *Lactococcus lactis* JC10 and kept in incubator at 37°C at static condition for 24 hour. After that the CFS was collected from each flask and examined for bioactivity to determine the bacteriocin production by spot on lawn method.

Likewise to achieve maximum bacteriocin production, *Lactococcus lactis* JC10 was inoculated individually in sterile production medium such as nutrient broth, luria broth, tryptic soy broth and MRS broth (control). Then all the flasks were kept in incubator at 37°C at static condition for 24 hour. After that the CFS was collected from each flask and examined for bioactivity against the indicator strains to determine the bacteriocin production by Spot on Lawn Method.

Results and Discussion

Effect of Incubation Temperature on Bacterial Growth and Bacteriocin Production

In any bioprocess, specific temperature requirement and its regulation is one of the most crucial parameter. Temperature plays a critical role in bacterial growth as well as bacteriocin production. It was observed from the experiment that maximum production has been achieved at 37°C showing an activity of 3200 AU/mL. At 27°C although the bacterial growth was not significantly lesser but the production was largely reduced giving an activity of 2400 AU/mL. This is the evidence that *Lactococcus lactis* JC10 is capable of producing bacteriocin at lower temperature also. This property can help it to be used as a preservative to be applied at cold temperature. Bacteriocin production although very little was detected even at 45°C as well as 10°C, but it lost its activity completely when incubated at 65°C. Similar result was reported previously by Ansari., *et al.* 2012. No bacteriocin production was found at 60°C.

Temperature (°C)	O.D (660 nm)	Inhibition Zone Diameter (mm)	Bacteriocin Activity (AU/mL)
10	0.322	4 ± 0.1	800 ± 20
27	1.287	12 ± 0.15	2400 ± 30
37	1.321	16 ± 0.15	3200 ± 30
42	1.229	12 ± 0.2	2400 ± 20
45	0.124	4 ± 0.2	800 ± 20
65	0.011	0 ± 0	0 ± 0

Table 1: *O.D values (660 nm) and Bacteriocin Activity at different temperatures.*

Effect of pH on Bacterial Growth and Bacteriocin Production

Maximum bacteriocin production was achieved at pH 6.8 showing an activity of 3200 AU/mL (Table 2). Production was also observed at pH 4.5 which is a slightly acidic condition. Bacteriocin production was observed at pH as high as 10.5 which is quite alkaline. Bacterial growth was reduced at alkaline pH. As the pH increases upto 12.5, a sharp decrease in bacteriocin activity was observed resulting in 800 AU/mL activity. So, pH 6.8 is found to be the optimum pH for maximum bacteriocin production. This wide range of pH tolerance makes this bacteriocin a suitable candidate for biopreservation of a broad range of acidic and non-acidic foods.

pH	$O.D_{660nm}$	Inhibition Zone Diameter (mm)	Bacteriocin Activity (AU/mL)
4.5	1.120	11 ± 0.2	1400 ± 40
5.5	1.221	13 ± 0.2	2600 ± 40
6.8	1.321	16 ± 0.15	3200 ± 30
7.5	1.110	14 ± 0.4	2000 ± 80
8.5	0.987	14 ± 0.25	1000 ± 50
10.5	0.871	10 ± 0.3	2000 ± 60
12.5	0.324	4 ± 0.3	800 ± 60

Table 2: *O.D values (660 nm) and Bacteriocin Activity at different pH.*

Effect of Different Preformed Media on Bacterial Growth and Bacteriocin Production

Lactococcus lactis JC10 when grown on readymade MRS media which is eventually the most widely used selective medium for the growth of Lactic Acid Bacteria (LAB), showed maximum bacteriocin activity which was found to be 3200 AU/mL. Nutrient broth, most commonly known universal media, also observed bacteriocin activity but it was lesser than that in MRS broth. One interesting finding was observed in Luria Broth which is also known as Lysogeny broth. Bacterial growth took place there but bacteriocin production was very less. Incidentally Luria broth is used as the industry standard for the cultivation of *Shigella* sp. and *Escherichia coli* generally. In trypticase soy broth, negligible amount of bacterial growth was observed with no bacteriocin production. Trypticase soy broth is known to support the growth of aerobic bacteria only and it supports LAB growth only when the media is supplemented with surfactants. So our findings are in accordance with the fact.

Media	$O.D_{660\ nm}$	Inhibition Zone Diameter (mm)	Bacteriocin Activity (AU/mL)
MRS (control)	1.321	16 ± 0.3	3200 ± 60
Nutrient Broth	0.987	12 ± 0.15	2400 ± 30
Luria Broth	1.022	7 ± 0.1	1400 ± 20
Trypticase Soy Broth	0.119	0	0

Table 3: *O.D values (660 nm) and Bacteriocin Activity when inoculated at Different Preformed Media.*

Optimization of Media Components

Effect of Different Carbon Sources at Different Concentrations

Maximum bacteriocin production was achieved with 4% lactose as the sole source of Carbon (3200 AU/mL) followed by 0.5% Glucose as the sole source of Carbon (2100 AU/mL). Other Carbon sources produced less bacteriocin (AU/mL) than these. Mannitol being the lowest bacteriocin producer followed by Galactose.

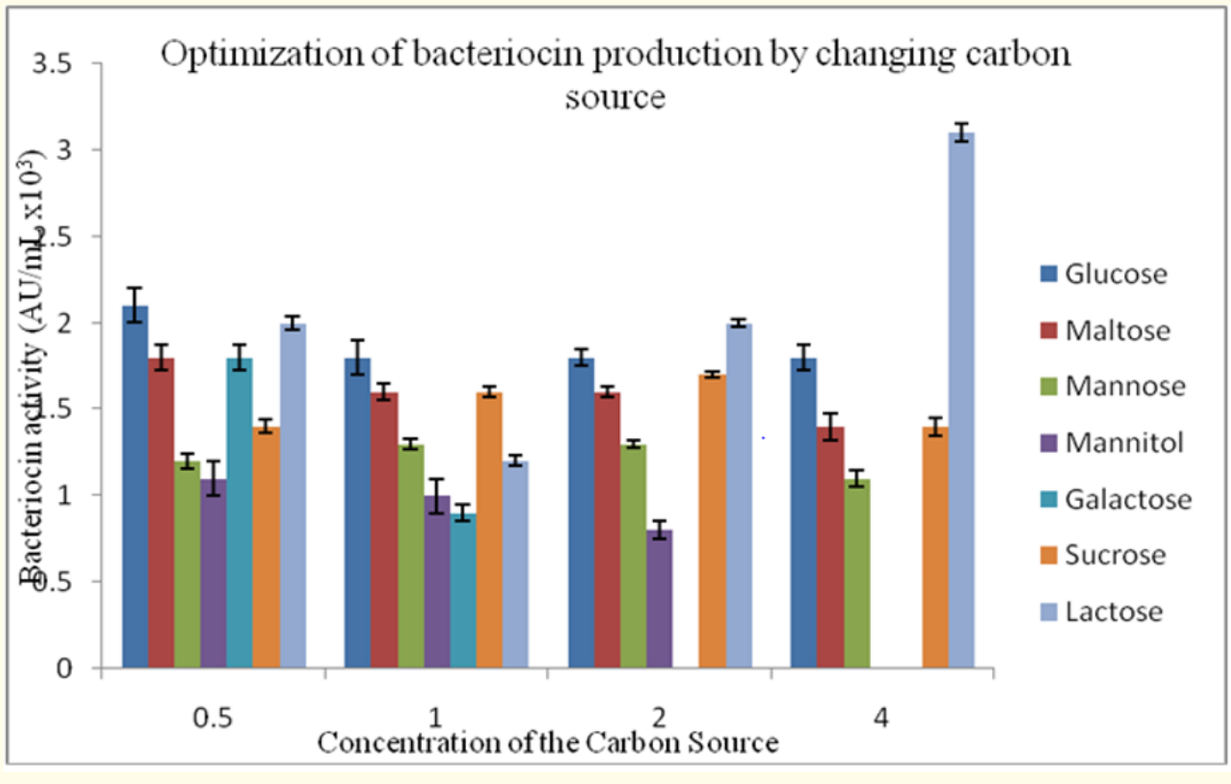

Figure 1: *Effect of Carbon sources on bacteriocin production. Control showed a value of 2.8 × 103 AU/mL.*

Effect of Different Nitrogen Sources at Different Concentrations

The effect of Nitrogen source on bacteriocin production revealed that Yeast Extract at 1.5% favoured maximum bacteriocin production (3200 AU/mL) and the minimum production was noticed in Beef Extract 1.5% (600 AU/mL). Yeast extract was found to be the most suitable Nitrogen source at all the 4 concentrations used (0.5%, 1%, 1.5% and 2%).

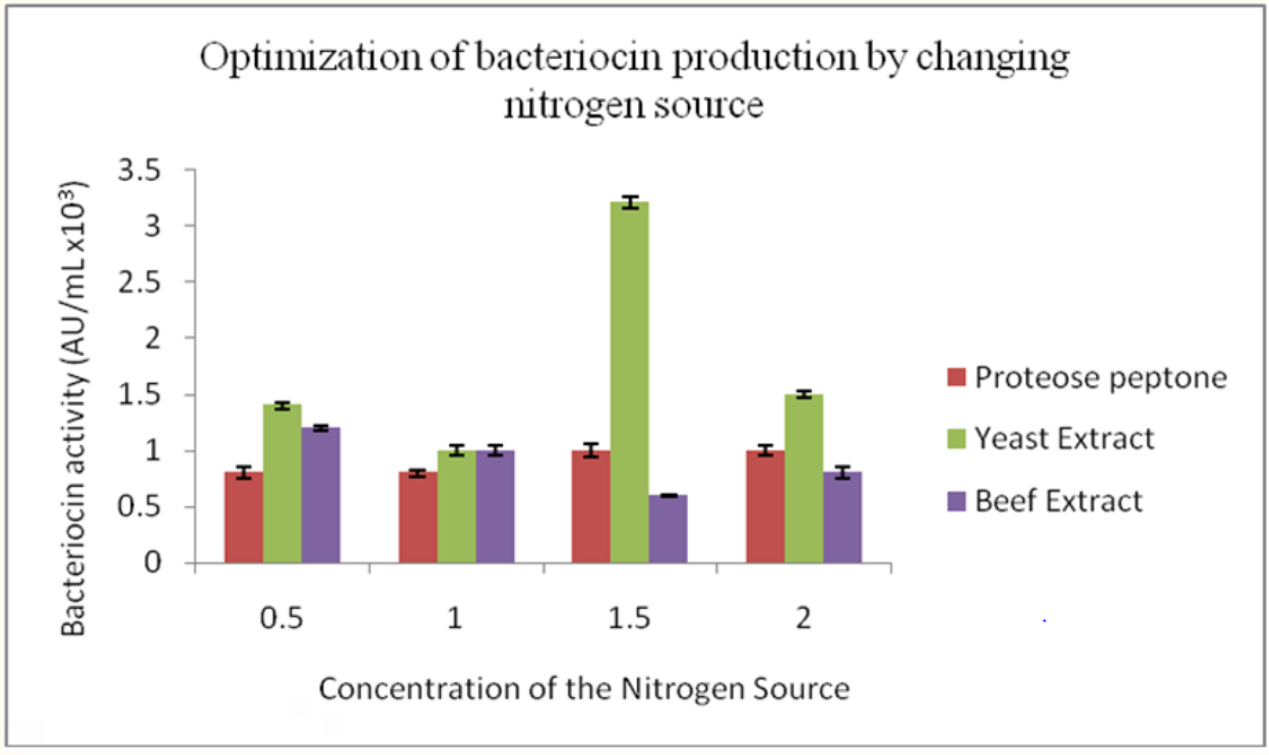

Figure 2: *Effect of Nitrogen sources on bacteriocin production. Control showed a value of 2.8 × 103 AU/mL.*

Effect of Different Surfactants at Different Concentrations

Surfactants play very important role in the growth media for cultivation of Lactic Acid Bacteria as it increases the surface tension and assists in nutrient uptake by Lactic Acid Bacteria. Maximum bacteriocin production was achieved with Tween 80 at 0.2% (3200 AU/mL) followed by Tween 80 at 0.1%. Minimum bacteriocin production was found with Tween 40 at 0.05%.

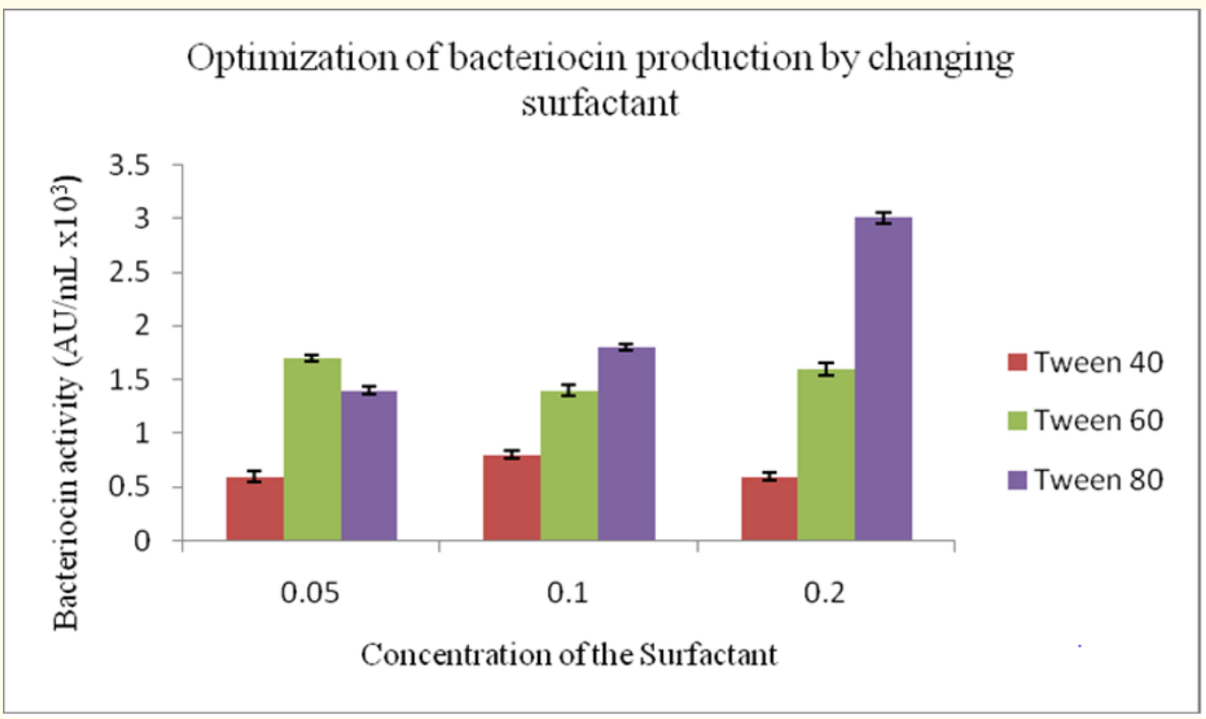

Figure 3: *Effect of Surfactants on bacteriocin production. Control showed a value of 2.8 × 103 AU/mL.*

Effect of Different Salts at Different Concentrations

Salts play very important role in MRS media for supporting the growth of Lactobacillus by suppressing the growth of other competing bacteria. Maximum bacteriocin production was achieved using Dipotassium Hydrogen Phosphate at 0.45% as sole salt source followed by the same salt at 0.3%. Minimum production was reported when sodium acetate at 0.1% was used as sole salt source.

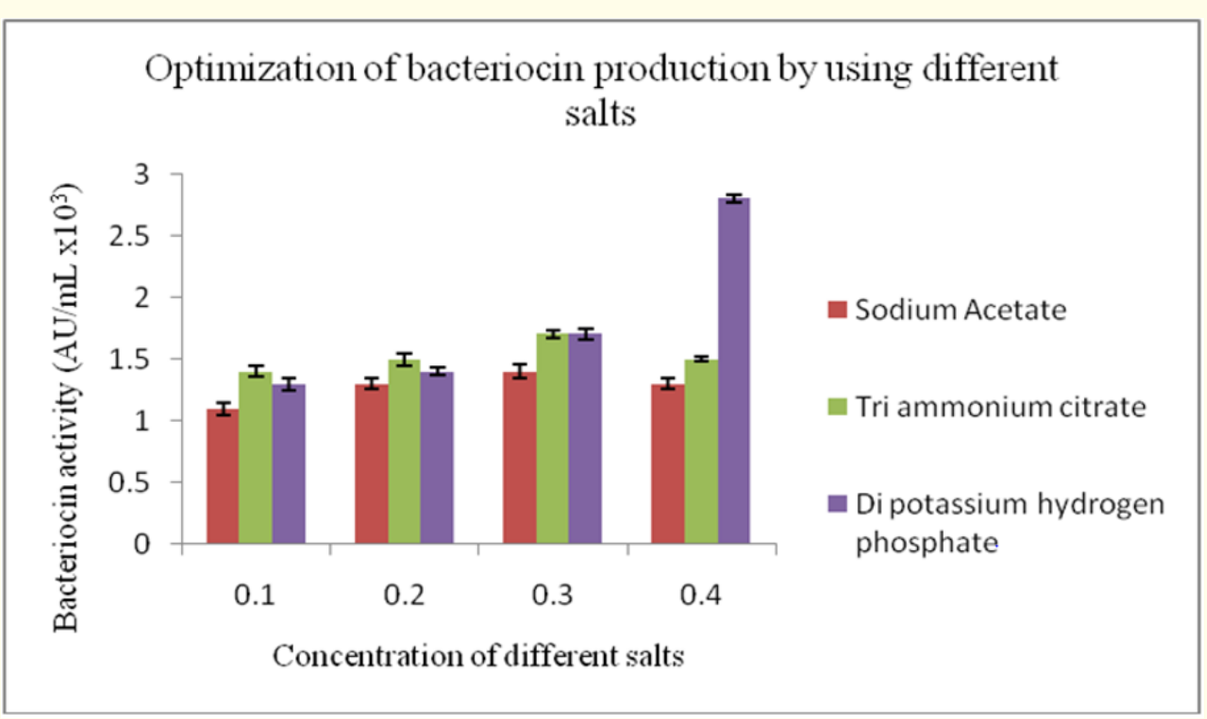

Figure 4: *Effect of salts on bacteriocin production. Control showed a value of 2.8 × 103 AU/mL.*

Conclusion

Bacteriocin produced by the candidate bacteria *Lactococcus lactis* JC10 showed antagonistic activity against a wide range of food borne pathogens. Bacteriocin activity was strongly dependent on pH, temperature and nutrient source. It's wide pH tolerance, activity retention in low and high temperature suggested it's wide applicability different food products. But for making this bacteriocin commercially viable and low cost, the media and culture condition have been optimized. These parameters are important for the optimization of growth and bacteriocin production, essential for the use of this strain or its bacteriocins as biopreservation agents for industrial applications. The optimization data on bacteriocin production provides the basic idea and information for carrying out statistical optimization method and commercial scaling up of the whole process.

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Volume 15 Issue 4 April 2019