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Optimization of Fermentation Conditions for Bacteriocin Production by *Lactococcus lactis* CCSULAC1 on Modified MRS Medium

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Abstract: The present study was carried out to optimized medium composition and culture conditions to enhance the bacteriocin production from *Lactococcus lactis* CCSULAC1 in MRS medium. The optimum temperature and initial pH for bacteriocin production was 7.5 and 35°C, respectively. *Lactococcus lactis* CCSULAC1 displayed the highest bacteriocin activity when grown in modified MRS medium containing soya extract (SEMRS) as nitrogen source instead of other selective constituents. Different culture media including MRS with 1.5% tween, TGEA, TGYA, Elliker's media were also studied for bacteriocin production. The bacteriocin showed broad antimicrobial spectrum determined by well diffusion assay against *E. coli*, *Enterobacter* sp., *S. aureus*, *Pseudomonas* sp., *B. polymyxa*, *B. subtilis*, *S. typhii*, *Micrococcus* sp. but shown no effect on *Candida albicans*, *Shigella* sp., *Klebsiella* sp. and *S. paratyphii*. Replacement of soya extract with yeast extract demonstrated the better yield of bacteriocin. The modified SEMRS is a cost effective medium that can be helpful for large scale industries.

Key words: Bacteriocin, modified SEMRS, *Lactococcus lactis* CCSULAC-1, antimicrobial activity

INTRODUCTION

Bacteriocins are natural product with potential in protecting food system against spoilage and pathogenic bacteria. They are antimicrobial peptides produced by many strains of lactic acid fermentation bacteria used in food processing (Conway, 1996; Messens and De Vuyst, 2002). Bacteriocins are widespread throughout the prokaryotic world and show diverse chemical and physical properties. Food-grade bacteria are constantly screened for new types of bacteriocins. The bacteriocins from lactic acid bacteria are small, heat stable, hydrophobic and cationic peptides (Jack *et al*., 1995). It is generally accepted that bacteriocins are a heterogeneous group of proteineous compound varying in activity spectrum, mode of action, molecular weight, genetic origin and biochemical properties (Ennahar *et al*., 2000; McAuliffe *et al*., 2001). The effect of pH and temperature are very important for bacteriocin production and have been studied in several lactic acid bacteria such as pediocin (Biswas *et al*., 1991), enterocin (Parente and Hill, 1992), lactococcin (Parente *et al*., 1993) and mesenterocin (Daba *et al*., 1993). Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is one of the most studied lantibiotics, due to its industrial application and potential for other uses egg fermented dairy products,

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inclusion of nisin in active packaging, use in combination with thermal and non-treatments. It is composed of 34 amino acids, a broad spectrum of activity and extreme heat stability and low pH (Delves-broughton *et al*., 1996). Nisin is generally recognized as safe (GRAS) and its production is affected by carbon, nitrogen and phosphorus regulation sources (De Vuyst and Vandamme, 1992, 1993). Although, other bacteriocin production is often performed in complex media, which promote abundant growth and relatively high bacteriocin levels, it seems more economical to use some of the waste from food industry as the raw material and some other less economic nutrient sources for culture media. The need to maximize results on raw material have encouraged new ways. Recently, soya waste and ensiled shrimp waste are good protein sources but low in dry matter and methionine content and high in chitin content. The best performance in terms of growth rate and carcass quality was found with replacement of up to 60% of soybean meal by soya waste which gave the lowest feed costs. In our previous studies, we evaluated the soya nutri nuggets extract medium for higher bacteriocin production (Kumari *et al*., 2008). In this study we evaluate the effect of temperature and pH in bacteriocin production on partially modified medium i.e., Soya Extract MRS (SEMRS) in which yeast extract is substituted with soya extract and to determine its antimicrobial profile.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacteriocin producing strains were isolated from different curd samples collected from local market of Meerut city in January, 2005. In all the cases, curd samples (stored at 4°C) were processed for isolation of Lactic Acid Bacteria (LAB) within 6-7 days. From sample collection to optimization of cultural conditions the whole project was completed within six months. Strain used for determining antimicrobial spectrum was obtained from Microbial type Culture Collection (MTCC Chandigarh, India). Before use in experiments, the stock cultures were activated by cultivation twice in nutrient agar medium (NAM) at 37°C as per instruction. *Lactococcus lactis* subsp. *lactis* MTCC3038 was used as the sensitive strain for the bacteriocin activity assay. Stock cultures were maintained at -70°C in MRS medium composition: peptone (10 g), yeast extract (5 g), glucose (20 g), disodium hydrogen phosphate (2 g), sodium acetate (5 g), triammonium citrate (2 g), $MgSO_4.7H_2O$ (0.02 g), tween80 (1 mL), agar (15 g), made to 1 L with distilled water, pH 6.5 (De Man *et al*., 1960) containing 20% (v/v) glycerol. All the AR grade chemicals used were purchased from qualigens and merck (India).

Isolation of Bacteriocin Producing Lactic Acid Bacteria (LAB)

We screened LAB strains from 50 samples of milk products comprising 22 samples of curd, 20 butter milk, 3 cheeses and 5 cream samples collected from different location of Meerut city, (U.P., India). Milk product (10 g) samples were aseptically transferred to 90 mL of sterile saline (0.9%) and mixed. Cheese samples were prepared by transferring 10 g of aseptically weighed sample to 100 mL sterile 2% sodium citrate solution at 45-50°C and homogenized for 3 min. In all the cases serial dilution were performed in sterile saline (0.9%). One milliliter sample by appropriate dilutions were spreaded uniformly on MRS medium. The plates were incubated at 37°C for 24 h and typical colonies were picked up randomly and transferred to MRS broth for microscopic examination and gas production subsequently. The plates were incubated at 37°C for 24 h. After 24 h typical colonies were picked up randomly and transferred to MRS broth for microscopic examination (gram staining, motility test) and

other tests including catalase test (Harrigan and Mc Cance, 1993), oxidase test gas production from glucose was assessed in nutrient broth containing 2% (w/v) glucose dispensed in test tube containing durham tubes. The composition of modified SEMRS is same as classical MRS except yeast extract was replaced with soya extract. Ten grams of soya beads were taken in 10 mL of distilled water boiled at 100°C for 15 min and filtered to obtain the Soya Extract (SE). The supernatant was filtered through 0.45 μm sterile membrane filter (millipore) and cell free filtrate obtained were stored in vials at -20°C. Supernatant was used for screening for bacteriocin production.

Bacteriocin Activity Determination

Inhibitory titers against the indicator bacteria were determined by agar diffusion well assay with slight modification (Yang *et al*., 1992). Log phase culture of bacteriocin *Lactococcus lactis* subsp. *lactis* (MTCC3038) was inoculated in 40 mL of sterilized modified SEMRS broth and incubated at 37°C for 24 h. One hundred microliter of broth culture was spread on MRS agar plates. Wells were cut with sterile cork borer (4 mm in diameter). Fifty microliter of CFF (cell free filtrate) which was serially diluted for two fold dilution was placed in each well. The plates were incubated at 37°C for 24 h, after incubation the plates were observed for clear circular zone of inhibition around the wells. The diameter of inhibition was measured with standard scale.

$$\text{AU mL}^{-1} = \text{Higher dilution that produce a distinct zone of inhibition} \times 1000\ \mu\text{L vol well}^{-1}$$

Selection of Medium for Bacteriocin Production

For selection of the medium, different media were tested individually including, MRS+ tween (1.5%), TYGA, TGEA, Elliker's broth and modified SEMRS (Yeast extract replaced by soya source). Erlenmeyer's flasks (250 mL) containing different medium (40 mL) was autoclaved for cultivation of bacteriocin producer. After inoculation with 1 mL of bacteriocin producing culture, they were incubated at 37°C at 150 rpm for 24 h. Bacteriocin activity was determined after 24 h as above.

Optimization of Temperature on Bacteriocin Production on Soya Extract MRS (SEMRS)

The inoculum was added in SEMRS broth and modified SEMRS broth in 250 mL erlenmeyer flask at different temperature 25, 30, 35, 40, 45 and 50°C containing 40 mL of culture medium in rotary shaker at 150 rpm for 24 h and absorbance at 600 nm (Systronic UV-VIS double-beam spectrophotometer 2201) was also determined for biomass production. Bacteriocin production was observed at different temperature against sensitive strain measured in terms of AU mL^{-1}.

Optimization of pH on Bacteriocin Production on Soya Extract MRS (SEMRS)

The inoculum was inoculated (1%) in SE MRS broth and modified SEMRS broth in 250 mL Erlenmeyer flask at different pH 5.0, 5.5, 6.0. 6.5, 7.0, 7.5 and 8.0 containing 40 mL of culture medium in rotary shakers at 150 rpm at 35°C for 24 h and absorbance at 600 nm (Systronic UV-VIS double-beam spectrophotometer 2201) was also determined for biomass production. Bacteriocin production was observed at different temperature against sensitive strain measured in terms of AU mL^{-1}.

Growth Determination and Protein Estimation

Growth (biomass) was measured by determining its turbidity of the culture in terms of OD (optical density) at 600 nm by spectrophotometer (Systronic UV-VIS double-beam

spectrophotometer 2201). Protein concentration in Cell Free Filtrate (CFF) was determined by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Antimicrobial Spectrum

The antimicrobial spectrum of CCSULAC1 was determined against different microorganisms. Exponential phase culture (12-18 h) of *Lactococcus lactis* CCSULAC1 (bacteriocin producer) was inoculated in 25 mL of sterilized SEMRS broth and incubated under optimum conditions, 20 mL of broth was then centrifuged (Sigma 2k15) at 15000 rpm for 30 min at 4°C. The supernatant was filtered through membrane filter of 0.45 μm and designated as Cell Free Filtrate (CFF). The test strains were spreaded on the NAM plate with the help of sterile swab and bored with help of sterile cork borer (4 mm) to obtain well. Fifty microliter of CFF which was serially diluted for two fold dilution and loaded in each well. The plates were incubated at 35°C for 24 h, after incubation, clear circular zone of inhibition were observed around the wells on the plates. The diameter of inhibition was measured with the standard scale and AU mL^{-1} is calculated as describe above.

RESULTS

Isolation of Bacteriocin Producing Lactic Acid Bacteria (LAB)

The bacteriocins appear to be a natural class of antibiotics distinguished from all others by sufficient properties to merit the distinctive name given to them by Jacob *et al.* (1953). Bacteriocins produced by various species of bacteria, in contrast to all other antibiotics, act only on strains of the same or closely related species. The empirical use of bacteriocin producing microorganisms or their natural products for the preservation of foods (bio-preservation) has been a common practice in the history of mankind (Ross *et al.*, 2002). The Lactic Acid Bacteria (LAB) produce an array of bacteriocins. Among all nisin (*Lactococcus lactis* subsp. *lactis*) was the only bacteriocin approved by FDA/WHO for the use of preservation. Bacteriocinogenic strains, although they possess the stable genetic ability to produce a bacteriocin, but do not do so under all conditions. From various milk samples and its products were collected (details in material and methods), among them 50 LAB isolate were randomly selected. Out of which, 15 were found to have antagonistic activity against sensitive strain (*Lactococcus lactis* MTCC3038). The selected strain was identified using on the basis of bergey's manual of systemic bacteriology (Holt *et al.*, 1994) to be *Lactococcus* sp., strain designated as *Lactococcus lactis* CCSULAC1 which exhibited strong antagonistic activity against sensitive strain and selected as bacteriocin producing strain used further to optimize condition in modified MRS.

Effect of Different Culture Media on Bacteriocin Production from *Lactococcus* sp. CCSULAC1

In order to improve the bacteriocin yield further, the effect of the yeast extract concentration on growth and bacteriocin production was tested using a modified MRS medium consisting of soya extract as nitrogen sources, as a substitute of yeast extract. The other medium like TGEA, TGYA and Elliker's broth were also studied which determines moderate activity and efficient biomass production. Table 1 shows the dependence of biomass and bacteriocin production in *Lactococcus lactis* subsp. *lactis* CCSULAC1 in different culture media. The strain displayed maximum activity i.e., 1250 AU mL^{-1} in MRS with 1.5% tween which is a classical medium for lactic acid bacteria. The MRS with soya extract (SEMRS) substituting with yeast extract determines better activity 1300 AU mL^{-1} with

Table 1: Bacteriocin production from *Lactococcus lactis* CCSULAC1 at 37°C in different culture media

Different culture media	Growth (OD_{600})	Activity unit (AU mL^{-1})	Specific activity (AU/OD_{600})
MRS	5.4	1200	222.22
MRS broth with 1.5% tween	5.4	1250	231.48
TGEA	5.3	1000	188.67
TGYA	5.4	1000	185.15
Modified MRS (SEMRS)	6.0	1300	200.00
Elliker's broth	5.3	900	169.81

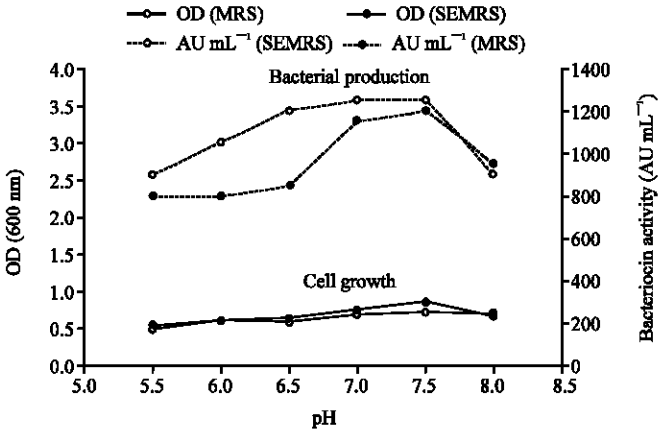

Fig. 1: Influence of initial pH on cell growth and bacteriocin production in MRS and SEMRS medium

increased biomass production as compared to classical MRS (1200 AU mL^{-1}). Similar observations have been made previously by Daba *et al.* (1993) in the production of meseteriocin. Biswas *et al.* (1991) compared to the production of pediocin ACH by *Pediococcus acidlactici* H cultivated in TGE broth, MRS broth and several modified media.

The Initial pH, Biomass and Bacteriocin Production

In parallel, pH is known to be important for biomass as well as bacteriocin production because aggregation, adsorption of bacteriocin to the cells and/or proteolytic degradation depend on pH and can affect the bacteriocin activity in *in vitro* culture conditions (Cheigh *et al.*, 2002; De Vuyst *et al.*, 1996; Parente *et al.*, 1994; Verellen *et al.*, 1998). The different ingredients of MRS medium have different effects on bacteriocin yield and biomass. For cell growth, glucose, peptone, yeast extract and potassium dihydrogen phosphate were positively significant factors, whereas for bacteriocin production, yeast extract was found to have significant effect of the former being negative and of the latter positive in our studies. The specific activity was found slightly higher on modified SEMRS i.e., 1363.6 AU mg^{-1} protein as compared to classical MRS 1214.16 AU mg^{-1} protein. *Listeria monocytogenes, Staphylococuss aureus* and *Pseudomonas* are frequent contaminants of raw milk in several countries (Asperger *et al.*, 1999). Therefore, searching for the natural substance with preventive and/or producing effect in the dairy product is of paramount importance. In our study along with previous reports produce bacteriocin in SEMRS showed better yields. Of course, more study is still needed to access the role of cost effective efficient supplements which play important role at large scale industrial fermentation processes.

Table 2: Initial and final pH of MRS, SEMRS on bacteriocin production from *L. lactis* CCSULAC1

	Final pH	
Initial pH	MRS	SEMRS
5.5	4.04	4.14
6	4.33	4.91
6.5	4.52	4.90
7	4.69	4.86
7.5	4.75	4.93
8	4.81	4.93

SEMRS: Soya extract MRS

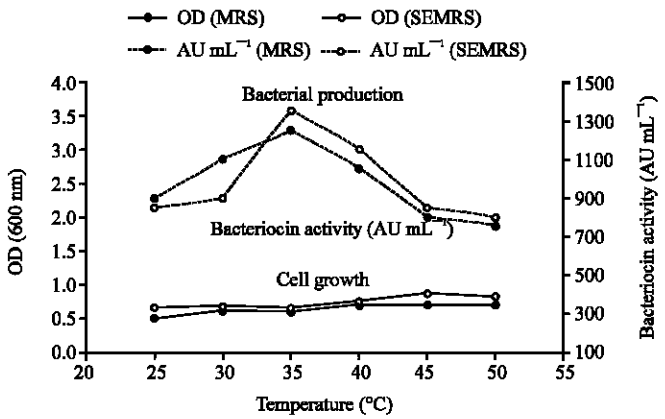

Fig. 2: Influence of temperature on cell growth and bacteriocin production in MRS and SEMRS medium

The *Lactococcus lactis* CCSULAC1 displayed constant growth at initial pH, below 5.5; the bacteriocin production of the strain was maximum when the pH was increased upto 7.0 and 7.5. Figure 1 shows the dependence of cell growth and bacteriocin production in *Lactococcus lactis* subsp. *lactis* CCSULAC1 on the initial pH of the MRS and SEMRS. Simultaneously, the difference observed in final pH (Table 2) was also found to be constant for MRS and SEMRS.

Effect of Temperature on Biomass and Bacteriocin Production

The effect of temperature on biomass and bacteriocin production by *Lactococcus lactis* CCSULAC1 was tested in shake flask cultures containing sterile MRS and SEMRS broth medium that was controlled at pH 7.5 and maintained at different temperatures (25, 30, 35, 40, 45 and 50°C). Figure 2 shows the biomass and bacteriocin production rate with an increase of temperature. The highest activity of bacteriocin (1280 AU mL^{-1}) was observed after at 24 h at 35°C. After, 40°C, biomass and bacteriocin activity decreases constantly with increase in temperature.

Antimicrobial Spectrum of CCSULAC1

Lactococcus lactis CCSULAC1 showed a wide inhibitory spectrum towards both gram-negative and gram-positive food spoilage and pathogenic bacteria. It inhibited 8 out of 12 test strains studied (Table 3). The *Lactococcus lactis* CCSULAC1 inhibits growth of *Bacillus polymxa, Bacillus substilis* MTCC441, *Staphylococcus aureus, Esherchia coli* MTCC119, *Salmonella typhii* MTCC734. At a same time no effect on growth of *Candida albicans, Klebsiella* and *Salmonella paratyphii* was also observed.

Table 3: Effect on bacteriocin production by *Lactococcus* CCSULAC1 at 37°C on NA medium different test organisms

Test Organisms	Sensitivity
Bacillus subtilis MTCC 441	+++
Bacillus polymyxa	+++
Staphylococcus aureus	+++
Escherchia coli MTCC 119	+++
Pseudomonas aeroginosa MTCC 2581	++
Micrococcus leutes	++
Salmonella typhii MTCC 734	+++
Enterobacter faecalis MTCC 439	++
Candida albicans	-
Salmonella paratyphii	-
Klebsiella sp.	-
Shigella sonnei MTCC 2957	-

+++: 16-14 mm, ++: 13-10 mm, -: No zone, NA: Nutrient agar

DISCUSSION

Bacteriocin-producers are lactic acid bacteria which need complex nutritions to grow and this not only increases the production cost, but also gives rise to the difficulties for their purification. Various media are used to cultivate the bacteriocin-producer such as CM (De Vuyst and Vandamme, 1992), SM8 (De Vuyst, 1995), M17 (Terzaghi and Sandine, 1975), M17S (Li *et al*., 2000) and MRS (De Man *et al*., 1960) media. All of these media are good for neutralizing lactic acid and improving cell growth, but do not consider the accumulation of bacteriocin and high content of nitrogen sources, especially proteins and peptides, that may bring about the difficulties of bacteriocin purification (Carolissen-Mackay *et al*., 1997). In the present study, we isolated lactic acid bacteria from fermented dairy products, among that the one of strain showed bacteriocin production which as designated as *Lactococcus lactis* CCSULAC1. Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is one of the most intensively studied lantibiotics, due to its industrial application and potential for other uses. It is composed of 34 amino acids and has a broad spectrum of activity and extreme heat stability at low pH. Nisin is generally recognized as safe (GRAS); it is commercially produced by microbial cultivation and has been widely used as a food preservative in many countries.

Growth of lactic acid bacteria is inhibited not only by the production of metabolites such as lactic acid but also by limited concentrations of indispensable nutrients present in the medium. Even in MRS, a commercial medium developed to support good growth of lactobacilli and often used for studying meat fermentations, inhibition of bacterial growth due to nutrient limitation occurs (Leroy and De Vuyst, 2001). As a result of the growth-associated character of bacteriocin production, this growth inhibition may lead to a limitation of bacteriocin production. Traditionally, optimization of bacteriocin fermentation processes has been performed by physiological metabolic control of their biosynthesis. Bacteriocins are usually produced in complex media (Biswas *et al*., 1991; De Vuyst and Vanddamme, 1992, 1993; Partente and Hill, 1992). Well controlled conditions of temperature and pH (Biswas *et al*., 1991; De Vuyst and Vandamme, 1992; Parente *et al*., 1994; De Vuyst *et al*., 1996) seem to play an important role in bacteriocin production. *Lactococcus lactis* CCSULAC1 produced bacteriocin in SEMRS (soya extract), which showed higher bacteriocin production than other studied medium in optimized fermenting conditions.

CONCLUSION

In present study, we introduce the cost effective substitute for nitrogen source i.e., soya extract, than the other medium used. The modified SEMRS medium can be further exploited on the large scale production of health promoters like bacteriocins.

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