

Full Length Research Paper

## Efficacy of plasmid curing agent on *Streptomyces longsporesflavns*

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Plasmid-specified traits like lactose metabolism and L-asparaginase production could be eliminated from *Streptomyces longsporesflavns* culture during production of fermented product (Microbial succession). In this study we mainly focus on plasmid profiling and plasmid characterization from *S. longsporesflavns* isolated from marine soil. Then we observe the efficacy of plasmid curing agent on *S. longsporesflavns*. Plasmid-free strains and cured derivatives harboring only a single plasmid (6.2 Kbp) were also obtained. Treatment of *S. longsporesflavns* with novobiocin at concentrations of 2.4 µg/ml could produce a large number of chloramphenicol<sup>r</sup> variants at a very high frequency (4.6%). These curing data confirmed the novobiocine act as an effective curing agent for *S. longsporesflavns*.

**Key words:** Antibiotic resistance, curing agent, *Streptomyces longsporesflavns*, plasmid profile, plasmid curing.

### INTRODUCTION

*Streptomyces* sp is a commercially important actinomycets variety of wide applications, both in food industry and as a probiotic agent for the improvement of human health, antileukaemia agent by secreting L-asparaginase (Cebeci and Gürakan, 2003). To improve the strain characteristic in food industries, genetic modification of *Streptomyces* strains are normally targeted toward the improvement or augmentation of specific strain characteristics, such as bacteriocin and L-asparaginase. Hence bacteriocin producing ability is mediated by plasmids; it is readily lost by plasmid

curing agents. For example, the gene for bacteriocin production is associated with the plasmid DNA in *L. plantarum* (Klaenhammer, 1978).

L-asparaginase belongs to an amidase group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonium. Asparagine is an amino acid required by cells for the production of protein. Asparagine is not an essential amino acid in normal cells and they synthesize this amino acid by the catalytic activity of asparagines synthetase from aspartic acid and glutamine. However, neoplastic cells

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cannot produce L-asparagine due to the absence of L-asparagine synthetase and they depend on cellular pools of L-asparagine for their growth. Tumor cells, more specifically, lymphatic tumor cells require huge amounts of asparagines for their rapid and malignant growth. L-asparaginase exploits the unusually high requirement tumor cells have for the amino acid asparagines (Sieo et al., 2005; Labeda et al., 2014) or *L. pentosus* (Whitehead et al., 2001). This enzyme has been isolated, purified and experimentally studied in detail as an antileukaemia agent in human patients and observed its high potential against childhood acute lymphoblastic leukaemia during the induction of remission or the intensification phases of treatment. Acute lymphoblastic leukaemia (ALL) is a malignant transformation of a clone of cells from the bone marrow where early lymphoid precursors proliferate and replace the normal cells of the bone marrow. It can be distinguished from other malignancies of lymphoid tissue by the immuno-phenotype of the cells.

Therefore, this study was undertaken to ascertain whether the loss of chloramphenicol (Chlo) resistance and bacteriocin production has any correlation with the loss of plasmid. This study also focuses on selecting an effective curing agent for *Streptomyces longsporesflavns*.

## MATERIALS AND METHODS

### Isolation and identification

Among ten production strains, the potential strain was *S. longsporesflavns* based on its physiological and biochemical characteristic. The colonies were cream, beige, little sticks and smooth round. The strain was gram positive rod. In liquid MRS broth produced uniform turbidity. It was homo-fermentative and it showed positive reaction in the fermentation of galactose, glucose, fructose, mannitol, lactose, sucrose and maltose but not with rhamnose. It did not produce catalase and amylase. It was resistant to biliary salt and produces H<sub>2</sub>S. Future confirmation was done by MIS.

### Plasmid profiling

#### Isolation of plasmid

Plasmid was isolated according to the method of O'Sullivan et al. (1993), Klaenhammer et al. (1978) and Maniatis et al. (1982). *S. longsporesflavns* was cultured in SD broth for 16 to 18 h at 37°C. 5 to 10 ml of overnight culture or  $6.0 \times 10^3$  was centrifuge at 5,000 rpm for 5 min. The pellet was resuspended in 200 µl of sol A (25% Sucrose containing Lysozyme-30 mg/ml) and incubated at 37°C for 15 min. After incubation 400 µl of sol B (3% SDS gm and 0.2N Sodium hydroxide) was added, mixed vigorously and incubated at 7 min. Then 300 µl of ice cold solution C was added (3 M Sodium acetate (pH-4.8), mixed vigorously and immediately centrifuge at 14,000 rpm for 15 min. Then, the supernatant was transferred to a fresh 1.5 ml centrifuge tube and 650 µl of isopropanol was added. It was centrifuge at maximum 15 min (4°C). Collected pellet was resuspended in 320 µl of sdH<sub>2</sub>O, 200 µl of sol E (7.5 M Ammonium acetate containing ethidium bromide-0.5 mg/ml) and 350 µl of sol F (phenol and

chloroform-1:1). It was mixed and centrifuge at maximum 5 min (4°C). The supernatant was transferred into fresh centrifugation tube and 1 ml of ethanol (-20°C) was added. It was centrifuge and the pellet was washed with 70% ethanol thrice and then the pellet was resuspended in 40 µl Tris EDTA buffer.

### Agarose gel electrophoresis

Agarose gel electrophoresis was performed by a horizontal gel electrophoresis system described by Maniatis et al. (1982), Thoma et al. (2012) with 0.6% agarose (Genei, bangloure, India) at 100 V for 90 min and Tris Acetate EDTA (TAE) (pH 8.0) as the running buffer. After the gel was stained in 0.5 µg ethidium bromide/ml, it was photographed by a MP-4 Polaroid camera (Biorad).

### Detection of antibiotic resistance phenotypes

#### Disc diffusion method

Antibiogram was done according to Kirby-Bauer method (Jones et al., 1985). Small discs containing different antibiotics or impregnated paper discs were dropped in *S. longsporesflavns* culture MHA agar plate, a nutrient rich environment in which *S. longsporesflavns* can grow. The inoculated plates were incubated at 37°C. The antibiotic will diffuse in the area surrounding each tablet and a disc of *S. longsporesflavns* lysis will become visible. Since the concentration of the antibiotic was the highest at the centre and the lowest at the edge of this zone, the diameter is suggestive for the minimum inhibitory concentration or MIC.

Here the antibiotics used were amikacin, ampicillin and chloramphenicol. If the diameter of zone of inhibition is greater than 10 mm, it is considered as resistant and if it is less than 10 mm it is considered as sensitive.

### Minimal inhibitory concentration for chloramphenicol

Minimal inhibitory concentration is regarded as the lowest concentration of antimicrobial agent which inhibits growth. Chloramphenicol was the antibiotics used in this procedure. The concentrations of these tested antibiotics ranged from 3.125 to 200 g/ml. An appropriate amount of antibiotic solution was separately added to the SD broth, in order to obtain the desired antibiotic concentrations. Tubes containing SD broth without antibiotics were used as a control. Each of the tubes were inoculated with a 16-h *S. longsporesflavns* culture at a 1% concentration (v/v), and were anaerobically incubated for 24 h at 39°C, which represents the optimal growth conditions of these strains. We also determined the minimum inhibitory concentration (MIC), which is the lowest concentration of antibiotic sufficient to completely inhibit the growth of the organism. As recommended by Jones et al. (1985), the observation of a faint haziness in the growth medium was not considered to represent growth, whereas the observation of definite turbidity was considered growth, as the drug had clearly failed to completely inhibit growth at that particular concentration. In addition to visual observations, we also evaluated bacterial growth with a spectrophotometer (CECIL, USA). Turbidity of less than 0.15 at an OD of 600 nm was considered "no growth", and turbidity of 0.15 or greater was considered "growth". All experiments were conducted three times, each with triplicate.

### Bacteriocin assay

Bacteriocin production in the parent cultures and its cured

derivatives were determined by the method of Castaman et al. (1993) and Fons et al. (1997). The cultures were spot inoculated on M17 338 plates and incubated at 32°C for 16 h. Agar was detached from the edges of the petri plate and turned over onto the lid. The back surface of agar was flooded with 7 ml soft Elliker agar or BHI agar containing 0.7 ml of 16-hold indicator culture. Zones of inhibition around the test culture were measured after incubation for 24 h at the optimum growth temperatures of the respective indicator strains.

#### Plasmid curing using chemical agents such as acriflavine, ethidium bromide, novobiocin and SDS

*S. longsporesflavns* was subjected to plasmid curing according to the method of Sieo et al. (2005). The chemical agents used for plasmid curing of *S. longsporesflavns* were acriflavine, ethidium bromide, novobiocin and SDS. Prior to curing we determined the sublethal concentrations of the four curing agents for *S. longsporesflavns*. The sub lethal concentration was defined as the highest concentration allowing for the detectable, reduced growth of a *S. longsporesflavns*. The sublethal concentrations of SDS, acriflavin and ethidium bromide for *S. longsporesflavns* were found to be in the range of 1.8 to 40.0, 5 to 11, 12 to 28 and 2.5 to 6.5 µg/ml, respectively.

*S. longsporesflavns* were subcultured every 24 h in SD broth containing a sublethal concentration of the respective curing agents. At appropriate intervals (6, 12, 18 and 24 days) the cultures were serially diluted then plated onto the SD agar. After 48 h of incubation under anaerobic conditions at 39°C, the emergent colonies were duplicated onto fresh SD agar containing 100 µg/ml of chloramphenicol. Colonies that failed to grow on the SD antibiotic plates after incubation were considered to have been cured and their duplicates on the SD agar were extracted and maintained in SD broth for further analysis. Respectively bacteriocin assay was performed and colonies that fail to produce inhibitory zone were also selected for further analysis. The curing rate (%) was calculated according to the following formula,

$$\text{Curing rate (\%)} = (B/A) * 100$$

Where, A = number of colonies formed on MRS agar; B = number of colonies formed on MRS antibiotic agar.

#### Plasmid analysis

Ten ml of six to eight h *S. longsporesflavns* cultures which failed to grow in antibiotic agar plate were used for plasmid extraction. The cells were harvested by centrifugation at 3000 × g at 4°C. 200 µl of freshly prepared lysozyme soln was added to the cell pellet. The subsequent plasmid extraction procedure was conducted according to the method described by O'Sullivan et al. (1993).

## RESULTS

*S. longsporesflavns* strains showed antagonistic activity against *S. longsporesflavns*. Among the strains tested, the *L. bulgaricus* strain was susceptible high; hence it was selected as indicator strain. Among ten pathogens tested *S. longsporesflavns* premier zone of clearance was noted as 3.1 mm (Table 1).

**Table 1.** Inhibitory activity of bacteriocin.

Test organism	Zone of clearance (mm)	Result
<i>Bacillus subtilis</i>	2.1	(+)
<i>Staphylococcus aureus</i>	2.9	(+)
<i>Lactobacillus bulgaricus</i>	3.1	(+)
<i>Salmonella typhimurium</i>	2.1	(+)
<i>Salmonella paratyphi 'B'</i>	2.5	(+)
<i>Escherichia coli</i>	2.5	(+)
<i>Klebsiella pneumonia</i>	3.0	(+)
<i>Serratia marcescense</i>	2.3	(+)
<i>Pseu aeruginosa</i>	2.3	(+)
<i>Vibrio cholera</i>	1.2	(-)

Positive (+) :Diameter of zone>2 mm; Negative (-) : Diameter of zone<2 mm.

#### Plasmid curing experience

In plasmid curing study we use two types of plasmid curing agent as novobiocin and ethidium bromide. Among the two agents we tested, novobiocin and ethidium bromide have curing activity of 4.6 and 3.3%, respectively. Of these curing agents, novobiocin acts as effective curing agent; it could be successfully cured, of their bacteriocin production (20.3 kbp plasmid) and ethidium bromide was able to cure the chloramphenicol resistance plasmid (6.4 kbp) (Figures 1 and 2). Novobiocin failed to eliminate bacteriocin production, even after prolonged sub-culturing (every 24 h for 28 h) in sublethal concentration of individual curing agent. This plasmid cured mutated strains, named *S. longsporesflavns* M which has been used for future studies.

#### Confirmation of cured derivatives

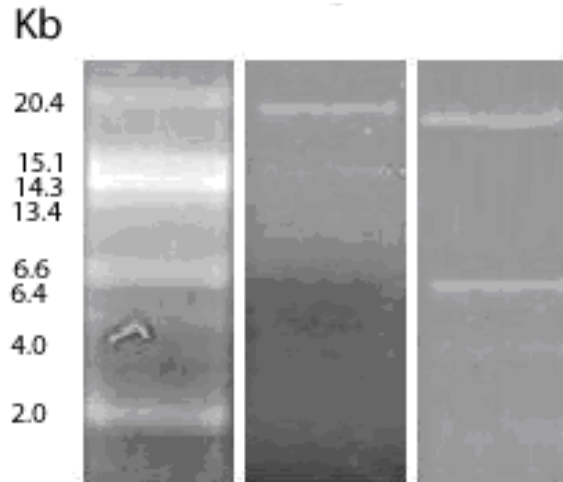
Confirmation of cured derivatives, antibiotic susceptibility was performed against *S. longsporesflavns*. In the present studies *S. longsporesflavns* M was sensitive against chloramphenicol. Whereas in bacteriocin assay it was negative against indicator strains (*L. bulgaricus*).

#### Optimization of physicochemical parameter

##### Growth characteristics of stress-treated cultures of *S. longsporesflavns*

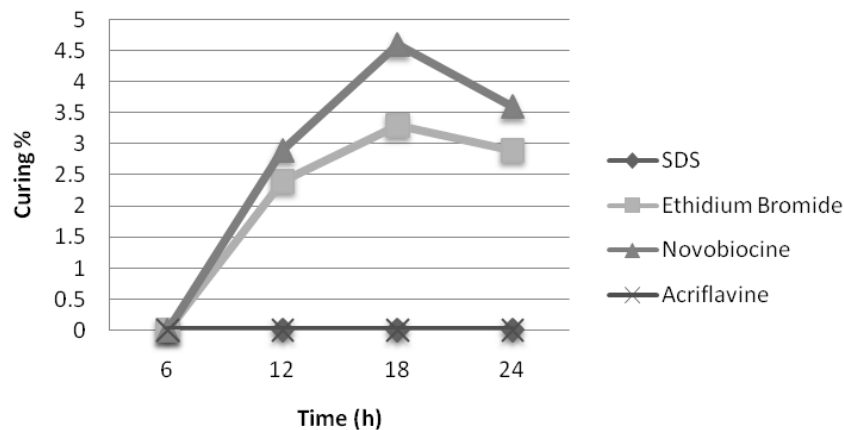
In the present study, an attempt was made to assess the stress tolerant capacity of *S. longsporesflavns* from marine ecosystem, such as temperature, salinity and pH.

Physicochemical parameters of the human marine varied according to the food habitat variations which in turn influenced the nature of the marine ecosystem. To



**Figure 1.** Plasmid profiles of wild type strains of *Streptomyces longsporesflavns* and their cured derivative (*S. longsporesflavns* M). Lane 1; super coiled standard DNA. Lane 2. *S. longsporesflavns* - cured derivatives (*S. longsporesflavns* M). Lane 2. *S. longsporesflavns* - wild strains.

### CURING EFFICIENCIES - CHLO PHENOTYPE



**Figure 2.** Curing efficiencies of Bac and Chlo phenotypes in *Lactobacillus acidophilus* MTCC447 after 24 h incubation.

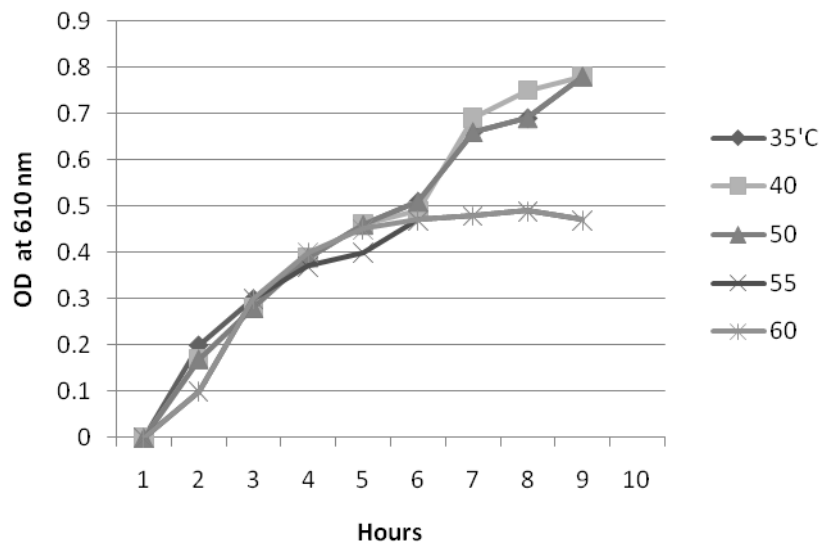
assess the stress tolerant capacity of *S. longsporesflavns* fr, we select the ecosystem based on the intestinal physiology, such as temperature, salinity and pH.

#### Temperature (°C)

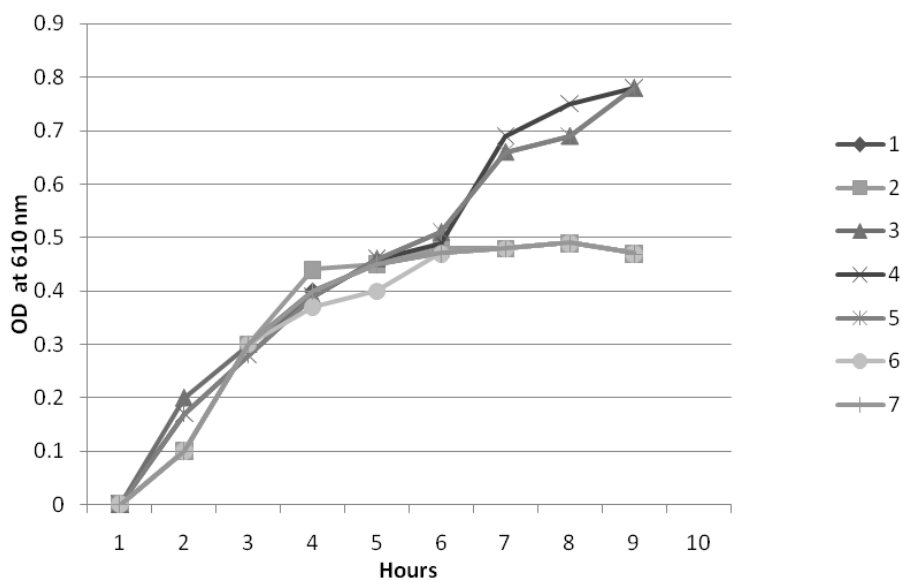
In the present investigation, the highest marine atmospheric temperature (AT) (60°C) was noticed and lowest (27°C) in Indian marine physiology was recorded. During the study, the average atmospheric temperature recorded was 28.95°C (Figure 3).

Based on this observation we select the temperature stress, such as the highest marine atmospheric temperature, 60°C and the lowest, 30°C.

The growth pattern was determined in different heat shock at 35, 40, 45, 50, 55 and 60°C during the exponential growth phase as monitored by OD<sub>610</sub> (Figure 1). Heat shock at 45°C for 30 min did not influence the growth rate of *S. longsporesflavns*. Heat shock at 50°C retarded the growth slightly (20% growth rate depressed) and the heat shock at 55°C retarded the growth rate severely (100% growth rate depressed). At 2 h after heat shock, the OD<sub>610</sub> value of the control and 50°C-shocked cultures were 0.8 and 0.7 respectively,



**Figure 3.** Growth characteristics of stress-treated cultures of *Streptomyces longisporiflavus*.



**Figure 4.** Growth characteristics of stress-treated cultures of *Streptomyces longisporiflavus*.

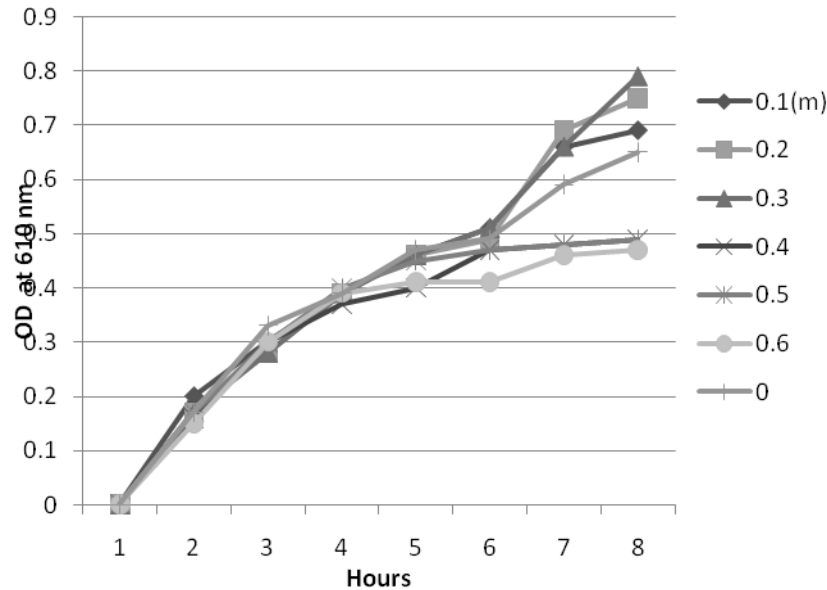
whereas the OD<sub>610</sub> of the 55°C-shock culture was only 0.54 (Figure 3).

### Salinity

*S. longisporiflavus* are often exposed to changes in the solute concentrations of their natural habitats like marine ecosystem. Nevertheless, their cytoplasmic solute concentration needs to be relatively constant (Kekessy et al., 1970). A sudden increase in the osmolarity of the environment (hyperosmotic stress) results in the movement of water from the cell to the

outside, which causes a detrimental loss of cell turgor pressure, changes the intracellular solute concentration and changes the cell volume. In the present investigation highest marine atmospheric osmolarity (3.2±3 mmol/kg) was noticed and lowest (1.32±4 mmol/kg) in Indian human intestinal physiology was recorded. During the study, the average atmospheric osmolarity recorded was 2.95±1 mmol/kg (Karthikeyan et al., 2009). Based on this observation we select the temperature stress, such as the highest marine atmospheric osmolarity, 1.0 and the lowest, 0.1 mol/kg.

In the present investigation, osmotic shock with 0.3 M NaCl did not affect growth (Figure 4), but higher salt



**Figure 5.** Growth characteristics of stress-treated cultures of *Streptomyces longsporesflavns*

concentration (0.4, 0.5, 0.6 and 0.7 M) reduced in the growth rate in the range of 12 to 40%. The osmolality of the MRS broth without any salt addition was  $390 \pm 2$  mmol/Kg, whereas addition of 0.6 M NaCl raised this value to  $1,498 \pm 2$  mmol/kg.

### pH stress

pH is an important environmental stress which occurs in LAB during fermentation of foods and beverages. The pKa of lactic acid is 3.86 and in its undissociated form it enters the cells by a carrier-mediated electroneutral process. At cytoplasmic pH, lactic acid dissociates which determines the stationary phase of growth, also if nutrients are still available (Roberts et al., 1968; Ramesh et al., 1987). Probiotic lactobacilli strains are exposed to extreme acid stress when they reach the stomach where hydrochloric acid is present. Several mechanisms regulate the homeostasis of  $pH_i$ . The proton-translocating ATPase is the most important for fermentative bacteria.

pH stress was selected based on marine ecosystem, the highest marine atmospheric pH was noticed (Sinha et al., 1981) and lowest in Indian human marine physiology was recorded. Based on this observation we select the pH stress, such as the highest intestinal pH 10 and the lowest, 2 (Figure 5).

The growth pattern of *S. longsporesflavns* was determined in different pH shock at 3, 4, 5, 6, 7 and 8 pKa during the exponential growth phase as monitored by  $OD_{610}$  (Figure 4). pH shock at 4 for 30 min did not influence the growth rate of *L. acidophilus* MTCC447. pH shock at 2 and 3 retarded the growth

slightly (20% growth rate depressed) and the pH shock at 7 retarded the growth rate severely (100% growth rate depressed).

## DISCUSSION

### *S. longsporesflavns* and antibiotic mutant

We assayed *S. longsporesflavns* strain with regard to their susceptibility to three antibiotics, namely chloramphenicol, erythromycin and tetracycline. This study focused primarily on these three antibiotics, because they serve as selective markers in transformation studies of actinomycetes (Kok et al., 1984). These antibiotics have also been listed as antibiotics which are authorized for veterinary medicine in Europe for the treatment of food-producing animals, including avian, bovine, porcine and porcine species (Charteris et al., 1998). The tested *S. longsporesflavns* exhibited varying degree of resistance to chloramphenicol. The MIC ranges from 50 to  $>100$   $\mu\text{g/ml}$  for chloramphenicol. Antibiotic resistance has been frequently found in *Streptomyces* species isolated from a variety of sources, chloramphenicol and erythromycin resistance have been found in *Streptomyces flavus* isolated from poultry (Robert et al., 1972; Varghese et al., 2015), whereas multiple-antibiotic resistance to cefoxitin, aztreonam, amikacin, gentamicin, kanamycin, streptomycin, sulphamethoxazole, trimethoprim, cotrimoxazole, metronidazole, polymyxin B and colistin sulphate has been observed in *Streptomyces* strains isolated from both the human GI tract and dairy products (Schwarz et al., 2001). The antibiotic resistance

profile of microorganism depends largely on previous exposure histories of the micro-organism, for example, to the type of antibiotics, period of exposure, and contact with other resistant microorganisms (Danielsen, 2002). Thus, antibiotic resistance profiles differ between microorganisms isolated from different sources. In the present study, *Streptomyces* strains were found to be sensitive highly to chloramphenicol (100 µg/ml) isolated from dairy industries. Antibiotic resistance can be attributed to enzymatic inactivation, decreased intracellular drug accumulation, a lack of target sites for antibiotic drugs, or the presence of genes that confer antibiotic resistance (Castaman et al., 1993; Fons et al., 1997), or within the bacterial genome.

Most actinomycetes species, regardless of their source (plants, meat, silage, sourdough or GI tract), harbour at least one indigenous plasmid (Adwan et al., 1998). The functions of these plasmids have classically been correlated with phenotypical properties, including drug resistance, carbohydrate metabolism, amino acid metabolism, and bacteriocin production. Although antibiotic resistance is frequently linked to plasmids, exceptional cases have also been reported. In the present study, the elimination of chloramphenicol resistance in *S. longsporesflavns* was associated with any plasmid loss. Nwosu et al. (1999) suggested that more than one antibiotic resistance mechanism might be present, due to their observation that not all antibiotic-resistant strains harbored plasmids. This hypothesis is in agreement with the results of Vescovo et al. [1982], Roberts et al. (2014), in which the loss of one plasmids from *S. longsporesflavns* via the curing process was found to be related to antibiotic resistance. Wegener et al. (1993) also determined that resistances to penicillin in 44 strains, and kanamycin in 15 strains of *Staphylococcus hyicus* were not related to plasmids. By way of contrast, the chloramphenicol resistance of *S. longsporesflavns* in the present study may have been conferred by one plasmid (20.3 kb) that was lost in the cured derivative. However, this will remain unconfirmable pending further analysis with regard to the characteristics of the plasmid. Most antibiotic resistance in *Streptomyces* strains has ultimately been determined to be plasmid mediated. Danielsen (2002) has located the gene conferring tetracycline resistance on *L. plantarum* 5057 in one of the four plasmids that are present in the strain. The erythromycin-resistant gene in *L. reuteri* and *L. fermentum*, and the chloramphenicol-resistant gene in *L. reuteri*, are both also associated with plasmids (Cebeci and Gürakan, 2003).

The plasmid under study was found to be refractory to various physical and chemical plasmid curing agents. One of the reasons could be that it may be carrying unknown functions vital for cell viability. This is more likely for bulky plasmids. For example, none of the megaplasmids of *Rhizobium* or *Agrobacterium* that exceed 250 MDa have yet been cured (Charteris et al., 1998). Interestingly enough, we found some metabolic activities in this bacterial strain, such as degradation of

salicylic acid or n-alkanes (C6±C18) which are so far reported to be carried on one or more well characterized plasmids such as the SAL and OCT plasmid respectively (Aghaiypour et al., 2001). Such metabolic markers with lower molecular weight have not previously been reported to be present in Bacteriocin production (6.2 kbp) and chloramphenicol resistant (20.3) by *S. longsporesflavns*. From these data it may be concluded that the phenotypic expression of chloramphenicol resistant genes is quite stable. Hence this strain may produce broad spectrum bacteriocin and it may be used as probiotic strain processes to work satisfactorily without spontaneous loss of bacteriocin production and chloramphenicol resistant degrading ability; the fate is often predicted for plasmid encoded markers. Hence, it can be concluded that novobiocin could be a very useful curing agent for the elimination of highly stable plasmids from *S. longsporesflavns* and thus can help us in the construction of new strains with desirable characteristics through their genetic manipulation (Rong et al., 2012).

### ***S. longsporesflavns* and bacteriocin mutant**

The present investigation highlighted the isolation, partial characterization and activity of bacteriocin produced by a *S. longsporesflavns* strain. Dairy industries seem to be a good source of actinomycetes. Among ten strains tested, the most potential strain was selected and used for further study. The physicochemical characterization of the strain revealed that it was *S. longsporesflavns*.

It was tested against 10 different bacterial pathogens which are usually present in food and can cause food borne illnesses in human beings. The bacteria selected were *L. bulgaricus*, *Salmonella typhimurium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella paratyphi 'B'*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. The result indicated that the present strain seemed to have antagonistic activity against nine pathogens in the order of *L. bulgaricus*, *S. typhimurium*, *B. subtilis*, *S. aureus*, *S. paratyphi 'B'*, *E. coli*, *K. pneumoniae*, *S. marcescens* and *P. aeruginosa*. The study had proved the possibility of using this strain as a probiotic.

Bacteriocins are antimicrobial agents produced by bacteria which are active against closely related bacteria (Balcao et al., 2001). They have been proved active against many other bacteria including pathogens (Danielsen, 2002). Hence they may be used as probiotics or as bio-preservatives especially in the acid fermentation of foods. In the present study an attempt was made to study the bacteriocin produced by *S. longsporesflavns* and the bacteriocin production was further studied in detail.

Bacteriocin production was strongly dependent on pH, nutrient source and incubation temperature (Schwarz

et al., 2001). Various physicochemical factors seemed to affect bacteriocin production as well as its activity. In the present study maximum activity was noted at pH 4, temperature 50°C and at 0.9% NaCl. The results obtained proved that it can be used in acidic foods like pickle, yogurt etc., as the optimum pH for activity was found to be pH 4.0.

The bacteriocin seemed to be a pure protein of about 2.5 KDa and it was found to be active at pH 4. Hence it deserves further study especially the molecular aspects. As maximum inhibitory activity was found in the culture medium at the stationary phase, it might be a secondary metabolite. MRS broth seemed to be a more suitable medium compared to GP broth for the bacteriocin production.

The use of bacteriocin-producing starter cultures for raw sausage fermentation may contribute to more uniform and safer products. However, the bacteriocin activity levels in a meat matrix are less than the expected activity levels. This is due to the specific conditions in the food environment. For this reason it is necessary to select strains that adapt to the meat environment. *L. sakei* CTC 494, an isolate obtained from Spanish fermented sausage and an organism that produces the anti listerial bacteriocin sakacin (Robert et al., 1972), was found to be able to exhibit maximum bacteriocin activity levels in the temperature and pH ranges which are typical for the fermentation stage of raw fermented sausages (Robert et al., 1968). However, how other factors, such as the presence of salt and curing agents, influence bacteriocin titers was unclear.

Previously, the effect of salt on production and/or activity of bacteriocins produced by lactic acid bacteria has been reported to be beneficial (Ali et al., 1994; Orsini, 2013) or harmful. In this study, using a mathematical model, we investigated how sodium chloride and acidic pH interfere with the kinetics of bacteriocin production by *S. Longsporesflavns* during *in vitro* fermentation. Due to its water binding and ionic characteristics, salt affects the metabolism of a starter culture. The growth of lactic acid bacteria is sometimes enhanced in the presence of low concentrations of sodium chloride (0.1 to 0.3 M), but growth is clearly inhibited in the presence of NaCl concentrations greater than 0.3M (wt/vol) (Lin et al., 1996; Endrz et al., 1999). Homofermentative LABs are more resistant to sodium chloride than heterofermentative LAB are, and strains resembling *S. longsporesflavns* have been shown to be more resistant than strains resembling *L. curvatus* (Siew et al., 2005) or *L. pentosus* (Whitehead et al., 2001). Increases in the salt concentration decrease the growth rate of *S. longsporesflavns*. Indeed, the growth rate often decreases linearly at water activity below the optimum level (Arima, 1964). Moreover, sodium chloride negatively affects the production of bacteriocin by *S. longsporesflavns*. Production of bacteriocin decreases because the amount of biomass formed decreases; bacteriocin production generally exhibits

primary metabolic kinetics (Pouwels et al., 1993; Derst et al., 1994) and because specific bacteriocin production decreases. It has been suggested that the decrease in bacteriocin production in the presence of salt is due to interference of sodium chloride molecules with binding of the induction factor, which is essential for bacteriocin production, to its receptor (Distasio, 1976). In the case of *S. longsporesflavns*, however, it appears that the water binding effect of salt molecules is the major factor responsible for the decrease in specific bacteriocin production since using glycerol as an agent to decrease water activity instead of salt has a comparable effect. Hence, because salt decreases water activity, the presence of relatively high salt concentrations in sausage batter may be one of the predominant factors. Combined inhibitory effects of sodium chloride and acidic pH on bacteriocin production reduce the efficacy of bacteriocin-producing starter cultures or co-cultures. During the fermentation stage, a salt concentration of 0.4 to 0.6M in the water phase of the sausage batter does indeed decrease bacteriocin production considerably. However, the activity probably is sufficient to have a significant anti-bacterial effect on the sausage environment, as demonstrated by Jones et al. (1985), Kak et al. (1984) and Blin et al. (2013). Moreover, the results of *in situ* experiments suggest that nitrite and pepper have a synergistic effect on the anti-listerial activity of *S. longsporesflavns* in sausage (Kekessyd et al., 1970).

pH is known mainly for its antimicrobial activity against sporeformers; it has a limited effect on the growth of lactic acid bacteria at acidic pH less than 2 (Datar, 1986), but at 4 inhibition is more pronounced (Castaman et al., 1993; Fons et al., 1997; Karthikeyan et al., 2009). It has been shown that biomass formation and bacteriocin production by *S. longsporesflavns* decrease as the concentration of pH increases. pH has no effect on specific bacteriocin production but decreases the bacteriocin titer indirectly because of its effect on cell growth. Since bacteriocin production is growth related, formation of a small amount of biomass results in a low bacteriocin yield. It has been mentioned previously that nitrite might interfere with active transport mechanisms. This would explain the surprisingly low  $V_{max}$  which is obtained when pH 4 is used. In this paper, we present a model that describes the combined effects of sodium chloride and pH on growth and bacteriocin production in *S. longsporesflavns*. The model accounts for a broad range of sodium chloride and acidic pH (0 to 8 and 2 to 4, respectively) in MRS broth at 25°C and 2% glucose conditions that are encountered during sausage fermentation. The predictive capacity of the model may be extended to other temperatures and pH values if the equations are combined with a previously described temperature- pH model (Ramesh et al., 1987; Sinha et al., 1991; Sieciechowicz et al., 1989). However, the accuracy of such a combined model approach needs to be evaluated. In this work, we examined the effects of



sodium chloride and acidic pH on bacteriocin production by *S. longsporesflavns*, a potential starter culture for sausage fermentation. Whereas temperature affected bacteriocin production only slightly because it decreased cell growth, salt had a more drastic effect because it decreased both cell growth and specific bacteriocin production. Addition of salt may be one of the major causes of the reduced efficacy of bacteriocin-producing starter cultures in food environments.

Bacteriocin is a bacterial substance, which is a biological protein moiety having bactericidal mode of action against the closely related species. Chemical analysis indicated that some bacteriocin, including that of *Staphylococcus*, *Clostridium* and *Lactobacillus* spp are quite complex molecules with lipid and carbohydrate components in addition to protein with lipid (Klaenhammer et al., 1996; Michael et al., 1981; Nwosu et al., 1999). Bacteriocins of actinomycetes are particularly important because of their essential role in the bacterial inhibition of majority of fermented foods. The present study showed that the bacteriocin is a simple protein moiety and the molecular weight of the bacteriocin was determined as 2.5 kDa.

Nisin is the first bacteriocin used as a food preservative agent in 1931. Nisin has first received approval by FDA to be used in pasteurized and processed cheese in 1988 (Wegener et al., 1993; Vescova et al., 1982). Like nisin the bacteriocin produced by *S. longsporesflavns* also has the potential to develop as a probiotic and can be used as a bio-preservative.

### Conflict of Interests

The authors have not declared any conflict of interest.

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