

Comparative Study on the Antimicrobial Activity of Probiotic *Bacillus subtilis* SK09 against Microbial Isolates from Dairy Effluent

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Abstract

Health promoting microorganisms such as Probiotics are recently been used as food additive and therapeutic supplement in order to enhance prophylaxis and digestion. Probiotic organisms with their ability to inhibit the growth of pathogens by producing antimicrobial peptides, so-called bacteriocin would be of an added therapeutic value. In the present study we report the isolation and partial purification of bacteriocin from spore forming probiotic culture of *Bacillus Subtilis* SK09 and tested for its activity to inhibit the growth and proliferation *E. coli*. This antimicrobial property was compared against *Lactobacilli* sp. Yeast and *Pseudomonas* sp. that are isolated from dairy effluent. The dilution at which the maximum activity of bacteriocin was determined based on *in-vitro* zone clearance method. Here we report that *Bacillus subtilis* SK09 and *Lactobacilli* sp. are the potential probiotic strains showing maximum activity even at 10^{-5} dilution.

Keywords: Probiotic bacteria; traveler's diarrhea; Zone clearance method; Antimicrobial activity.

Introduction

In the recent years Probiotic based products have gained a lot of attention due to their health promoting prospects. Probiotics are live microorganisms which when administrated in adequate amounts confer to health benefit or nutrition of the host by improving the intestinal microbial balance (Fuller, 1994).

Bacteria, such as enterotoxigenic *E. coli*, *Salmonella* sp. and *Shigella* sp., are the common causes of traveler's diarrhea, which affects millions of people traveling to developing countries each year (Yates *et al.*, 2006). Probiotic consumption has been proven to decrease the incidence of such disease, and also able to inhibit the growth of several pathogens both *in vivo* and *in vitro*, survives the gastrointestinal tract, and most importantly stay unaffected by antibiotic therapy (Boddy *et al.*, 1991). Moreover certain probiotic bacteria have been reported to produce inhibitory compounds called Bacteriocin, proven to be antagonistic to various degrees against intestinal pathogens. (Gibson and Wang 1994; Pessi *et al* 1999; Todariki *et al.*, 2001). Because bacteriocin are natural products of many micro organism associated with foods, there is currently enhanced interest in their use as natural food preservatives. Hence inhibition of the growth of pathogens by producing antimicrobial peptides, so-called bacteriocin, is considered as the highly appreciated probiotic effects (Diep *et al.*, 2009).

In our study we have successfully attempted to isolate microbial strains such as *Lactobacilli* sp., Yeast and *Pseudomonas* sp., from dairy effluents, since microorganisms isolated from dairy effluents are generally regarded as safe (GRAS). These organisms were assayed for its capability to produce bacteriocin and its inhibitory effect was checked

against growth and proliferation of *E. coli* strain. A comparative study on antimicrobial property had been conducted with spore forming, lactose fermenting probiotic novel isolate of *Bacillus subtilis* SK09 which was isolated previously by us. (Sreekumar and Soundarajan, 2010 a). The dilution at which the maximum activity of bacteriocin was determined, which could be attributed to the therapeutic usage of probiotics against various microbial infections in human.

Materials and Methods

The Primary clarifier effluent, generated at Aavin Dairy industry, Chennai was collected in sterile sampling vials. 1ml of the above sample was serially diluted in 9ml of sterile 0.8% saline. Dilutions up to 10^{-7} were achieved and plates corresponding to 10^{-3} , 10^{-5} and 10^{-7} were plated on Nutrient agar plates (M1362, HiMedia India). The Plates were incubated at room temperature for 24h and were observed for Colony forming units. Each distinct colony was isolated and plated separately as pure cultures in Nutrient agar medium.

The experimental strain *Bacillus subtilis* SK09 was isolated previously and characterized by us (Sreekumar and Soundarajan, 2010 b). The cultures were maintained in Nutrient agar slants at 4°C. 24 h prior to the day experiment the cultures were thawed to room temperature. Nutrient broth as Primary inoculum was prepared in aliquots of 10ml and sterilized in an autoclave at 121°C and 15psi; these were inoculated with one loopful of experimental organism from nutrient agar slants. The cultures were grown overnight at 32°C at 150 rpm. The isolated pure cultures from above method were subjected to morphological studies and biochemical tests as recommended by identification key proposed previously (Reva *et al*, 2001).

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Bacillus subtilis SK09, *Pseudomonas* sp., were grown in nutrient broth (M1362, HiMedia) whereas *Lactobacilli* sp., and Yeast was grown using MRS media (M369, HiMedia) and YPD media (M1363, HiMedia) respectively. The corresponding medium were prepared for prescribed concentration and sterilized in an autoclave at 121°C and 15 psi 20 min.

Duplicates of 200 ml corresponding broth was prepared in a 1000 ml Erlenmeyer flask with sterile distilled water and adjusted to pH 7.0, then sterilized in an autoclave at 121°C and 15 psi. The broth was cooled in a laminar air chamber and inoculated with 1% v/v of primary inoculum. Each of these flasks was placed in a rotary shaker maintained at 32°C for 24 h and 150 rpm. Periodical samples were withdrawn under sterile condition and analyzed for the microbial content by optical density at 600nm using UV-Visible spectrophotometer (Systronics 2201, India). After analyzing the residual substrate concentration using GOD/POD kit (Medox, India) (Anjan basak, 2007), the growth kinetic parameters such as specific growth rate (μ), doubling time (t_d) and yield coefficient (Y_{xs}) were determined. The culture broths were harvested at early stationary phase and were centrifuged in sterile 50 ml round bottomed centrifuge tubes at 6000 rpm for 15 min and 4°C. The cell free supernatant was collected in sterile 100 ml polystyrenes storage vials and stored at 4°C. To precipitate bacteriocin, ammonium sulphate was added to supernatant fluid to a fluid saturation of 55% and the final mixture was kept at 4 C for 20 hr. After centrifugation at 6000 rpm for 20 min, the pellet was suspended in 1/100 of original culture volume of SP buffer (25 mMol lit⁻¹ sodium phosphate buffer, pH 7.0). This solution was designated as the crude bacteriocin preparation (Zheng *et al.*, 1999. Kuramitsu *et al.*, 2005).

The antimicrobial activity of cell free crude extract was assayed using agar diffusion method. The targeted pathogenic organism, *E. coli* was grown over night at 37°C on M9 minimal salt, 5X agar media which has the following composition: Disodium Phosphate anhydrous (33.9 g/l), Monopotassium Phosphate (15.0 g/l), Sodium Chloride (2.5 g/l), Ammonium Chloride (5.0 g/l) along with suitable carbon source (Difco media). The cell free crude extract of bacteriocin was serially diluted to 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ using sterile SP buffer. 100 μ l of this diluted extract was added to the previously made wells of agar plate. These plates were incubated at 37°C for 24 hr. The diameter of zone clearance was measured and correlated with the dilutions of the sample (Sharma *et al.*, 2008, Kuramitsu *et al.*, 2005).

Results and Discussion

From dairy effluent, a total of four microbial isolates were identified such as *B. subtilis*, *Lactobacilli*, *Pseudomonas* and Yeast. The morphological and biochemical characteristics were analyzed for the genus identification. Among these isolates *Bacillus subtilis* SK09 was reported already by us as a probiotic strain with spore forming, lactose fermenting ability.

Growth kinetics is the relationship between specific growth rate and the concentration of a substrate and is one of the basic tools in microbiology to study the doubling time of a micro organism (Karin Kovárová-Kovar *et al.*, 1998). The growth pattern of each isolates and yield coefficient trends were plotted against time (Figs 1 & 2). The values of growth kinetic parameters (Table-1) resulted that *B. subtilis* SK09 strain showed higher values when compared to other strains. Bacteriocin is a secondary metabolite, its production starts at the onset of stationary phase of growth (*et al.*, 2004). On the basis of the growth pattern of isolates, early stationary period was ascertained and the biomass harvested after 350 min of incubation for bacteriocin extraction.

The targeted coliform organism *E.coli* had been grown using M9 minimal salt 5X agars medium, because it is necessitated to check the bacteriocin activity in the presence of salt concentration to ascertain its probiotic value even at intestinal environment (Lone Gram *et al.*, 1999). Antimicrobial activity assay against *E.coli* using zone clearance method proved that the activity of *B. subtilis* SK09 and *Lactobacilli* sp. were maximum with 18 mm and 15 mm diameter respectively even at 10⁻⁵ dilution (Figs 3 & 4). Even though the inhibitory activity of *Pseudomonas* sp. found higher in the initial dilutions, there was a clear loss in the activity at higher dilutions (Fig-5). In the case of yeast, the inhibition zone was found only 17mm even at 10⁻¹ dilutions, which proved its lowest antimicrobial activity when compared to other isolates (Fig-6). It was also determined that *B. subtilis* retained 60% of its initial activity even at 10⁻⁵ dilution, where as *Lactobacilli* retained 51% of its activity. The activity retained by *Pseudomonas* and Yeast were very low comparatively with only 17.54% and 16.66% respectively (Fig 7).

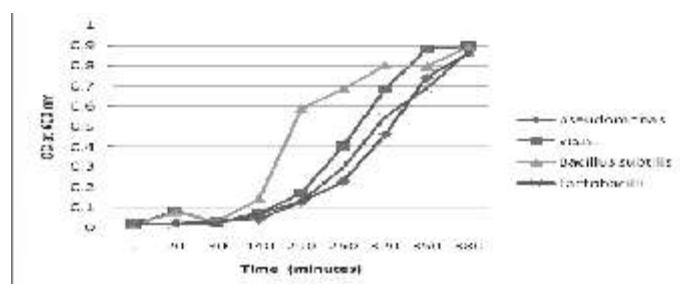


Figure 1. Plot showing Growth pattern OD_{600nm} Vs time

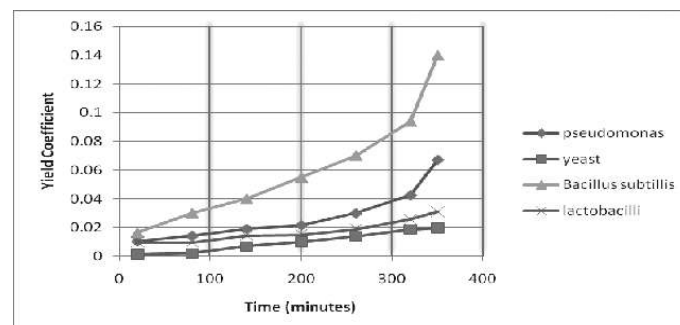


Figure 2. Plot showing Biomass Yield coefficient Vs time

Microbial isolate	Specific growth rate (μ min ⁻¹)	Doubling time (t_d , min)	Yield coefficient (Y_{xs})
<i>Bacillus subtilis</i> SK09	0.0257	26.955	0.06364
<i>Lactobacilli</i> sp.	0.0116	59.344	0.01778
<i>Pseudomonas</i> sp	0.0109	63.35	0.02914
Yeast	0.0106	64.98	0.0089

Table 1. Growth parameter values for microbial isolates

Conclusion

The experiment evidently proves that probiotic *Bacillus subtilis* SK09 strain is capable enough to produce antimicrobial compound that can show higher potency against *E. coli* even in the presence of salt medium. This novel strain can be used individually or as a mixed culture with other lactic acid bacteria to exert cumulative antimicrobial property.

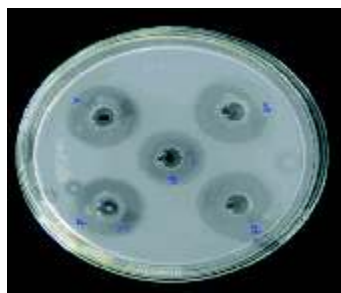
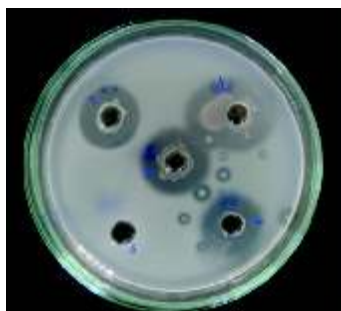
Figure 3. Antimicrobial activity of *B. Subtilis* Sk09Figure 4. Antimicrobial activity of *Lactobacilli* sp.Figure 5. Antimicrobial activity of *pseudomonas* sp

Figure 6. Antimicrobial activity of Yeast

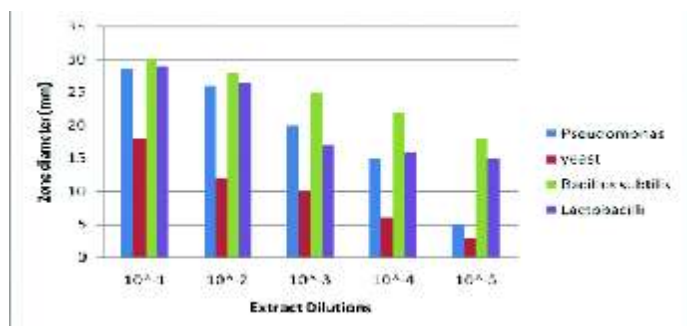


Figure 7. Bar chart showing Zone clearance diameter Vs dilutions

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