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Isolation and characterization of probiotic *Bacillus subtilis SK09* from dairy effluent

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Abstract

Health promoting microorganisms such as probiotics are recently been used as food additive and therapeutic supplement especially as enhancer of prophylaxis and digestion. Here we report the isolation and characterization of a probiotic bacillus species, with the ability to ferment lactose, from dairy effluent. Biochemical test and 16S rRNA sequencing were done in order to establish the species and strain details. The species was found to be *Bacillus subtilis SK09* with a unique ability to ferment lactose. The strain was also subjected to commonly available antibiotics for its antibiotic susceptibility for its probiotic credibility.

Keywords: Probiotic bacteria, *Bacillus subtilis*, lactose intolerance, 16SrRNA sequencing.

Introduction

Recent developments in the field of microbiology as led to the use of human friendly microorganism such as probiotics for the consumption as food additive and a therapeutic (Conway *et al.,* 1987; Fuller, 1994; FAO/WHO, 2002; Donkor *et al.,* 2006). 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; FAO/WHO, 2002). Various bacterial strains such as: *Bifidobacterium, Lactobacillus* and *Streptococcus* genera as well as yeast of *Saccharomyces* species. The majority of commercially available probiotic formulations contain *Lactobacilli* as opposed to *Bifidobacteria* as the former are more tolerant and more stable in food products. But *lactobacilli* viability in the stomach is greatly affected by its inability to form spore and endure the acidic environment of the stomach (Crittenden *et al.,* 1996; Begley *et al.,* 2006). Therefore the use of *Lactobacilli* as a probiotic is limited to the confines of it being used as the therapeutic prophylaxis alone rather than being a digestion enhancer.

The term lactose intolerance describes the inability to digest significant amount of lactose resulting from the shortage of enzyme lactase, which is normally produced by the cell that line the walls of small intestine (Kim *et al.,* 1983; De Vrese, 2001). It is estimated that over 75% of adults worldwide incur lactose intolerance (LT) or lactose malabsorbtion as they get older (Pribila *et al.,* 2000). Probiotics which can help LT people were developed by various researchers in the past decades like *Lactobacillus delbrueckii* (Lin *et al.,* 1998), which lacked the efficiency to colonize the lower gut because of their inability to form spores. Studies testing the multi-probiotic product VSL#3 also fail to improve mal-digestion (Szilagyi *et al.,* 2007). The case of lactose malabsorbtion is persistent problem for the development of potentially successful probiotics which should help the aforementioned people. Therefore it is the need of the hour to isolate a bacterial strain which is capable enough to be used as a probiotic that can ferment lactose and to have the ability to form spores. A probiotic which can be given as a supplement to lactose intolerant people to enhance their digestion of dairy products would be of a good economic and therapeutic value.

In this study we have successfully isolated a new strain of spore forming *Bacilli* that is capable of fermenting lactose from dairy effluents, since microorganisms isolated from dairy effluents are generally regarded as safe (GRAS). The organism was isolated and tested for its lactose fermenting ability through X-gal plates and β-galactosidase assay. The isolates were tested for its phenotypic and genotypic characteristics; they were subjected to an array of common antibiotics in order to analyze their probiotic characteristics. Further, 16SrRNA sequencing was performed in order to establish phylogenetic relationship and species specificity.

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Materials and methods

The primary clarifier effluent generated at Aavin dairy industry, Chennai was collected in sterile sampling vials. 1 ml of the above sample was serially diluted in 9 ml 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 infused with 50 µl X-gal (5-bromo-4-chloro-3-indoxyl-*β*-d-galactopyranoside) in order to select colonies with lactose fermenting ability. 20 mg of X-gal (Chempure, India) in 1 ml of N, N-dimethyl formamide solution was used for the above plate. The plates were incubated at room temperature for 24 h. After 24 h the plates were observed for CFUs with blue colourations indicating the presence of *β*-galactosidase producing organisms. Each blue colony was isolated and plated separately as pure cultures in nutrient agar medium. In order to isolate only spore forming bacteria from the lactose fermenters one loopful of pure culture of isolates were then inoculated to 50 ml of sterile Difco sporulation medium (DSM) (Nicholson & Setlow, 1990) in 250 ml erlenmeyer flask and maintained at 37°C for 48 h at 150 rpm. Each of the cultures was tested for spore formation under microscopic observation using Schaeffer-Fulton staining technique (Harley & Prescott, 2002). Those strains with spore forming ability further characterized for genotyping and phenotyping studies.

Phenotyping

The isolated pure culture from above steps was subjected to morphological studies and biochemical tests as recommended by Sneath *et al.* (1986). An identification key proposed by Reva *et al.* (2001) was used for identification.

16S rDNA sequencing

Genomic DNA from the above isolated strain was prepared as described by William *et al.* (2000). The 16S rDNA gene fragments were amplified by PCR using:

16s forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3'

and

16s Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3'

In an automated thermo cycler (eppendorf mastercycler) which amplify the maximum number of nucleotides in 16S rDNA from a wide variety of bacterial taxa (Hyronimus *et al.,* 1998). PCR reaction and DNA sequencing were performed by William *et al.* (2000) in an automated gene sequencer (ABI 3130 genetic analyzer). Databases (GenBank) were searched for sequences similarly to the 16S rDNA sequences obtained and a phylogenetic tree was established in correlation with other closely related bacterial stains (Weisburg *et al.,* 1991; Wiley *et al.,* 1991).

Antibiotic resistance analysis

Antibiotic susceptibility for strains were analyzed by using the disc diffusion method according to the recommendations of the national committee for clinical laboratory standards (Wayne, 1997). Cells from the 48 h old cultures were diluted1:20 and 100 µl was seeded on nutrient agar plates using a swab. Antibiotic-impregnated discs (Himedia, India) were placed on seeded plates and the zone of growth inhibition was measured after 24 h of incubation at 37°C.

Fig. 1. Blue-white colonies

Results

Isolation and characterization of probiotic Bacillus subtilis SK09

Clear blue colonies of were observed (Fig. 1) on the X-gal plates indicating the presence of lactose fermenting organisms. Individual colonies cultured in sporulation media resulted with only one strain with spore forming ability. The morphology of the above strain of bacteria was: individual motile, gram positive *Bacilli*, with elliptical endospores which formed flat and circular colonies with undulate margins. The biochemical tests on pure culture of the above strain revealed that it is aerobic organism showing positive results on oxidase and catalase test. The organism was able to metabolize glucose into organic acids and hence gave positive results for methyl red test. In the same way the organism does use butylene glycol pathway and thereby production of acetoin was

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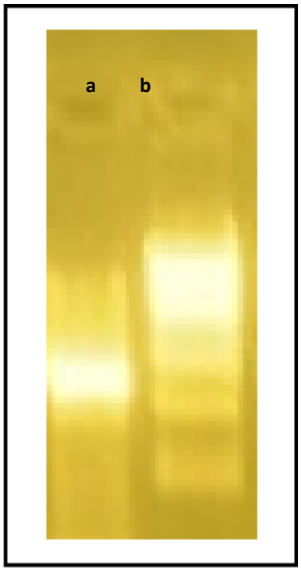
observed in Voges proskauer test. The organism can utilize citrate as a substrate and test positive for citrate test. The organism also has an ability to convert nitrate to nitrite and lacks the ability to convert tryptophan into Indole.

Fig. 2. a. Amplified PCR product, b. Ladder

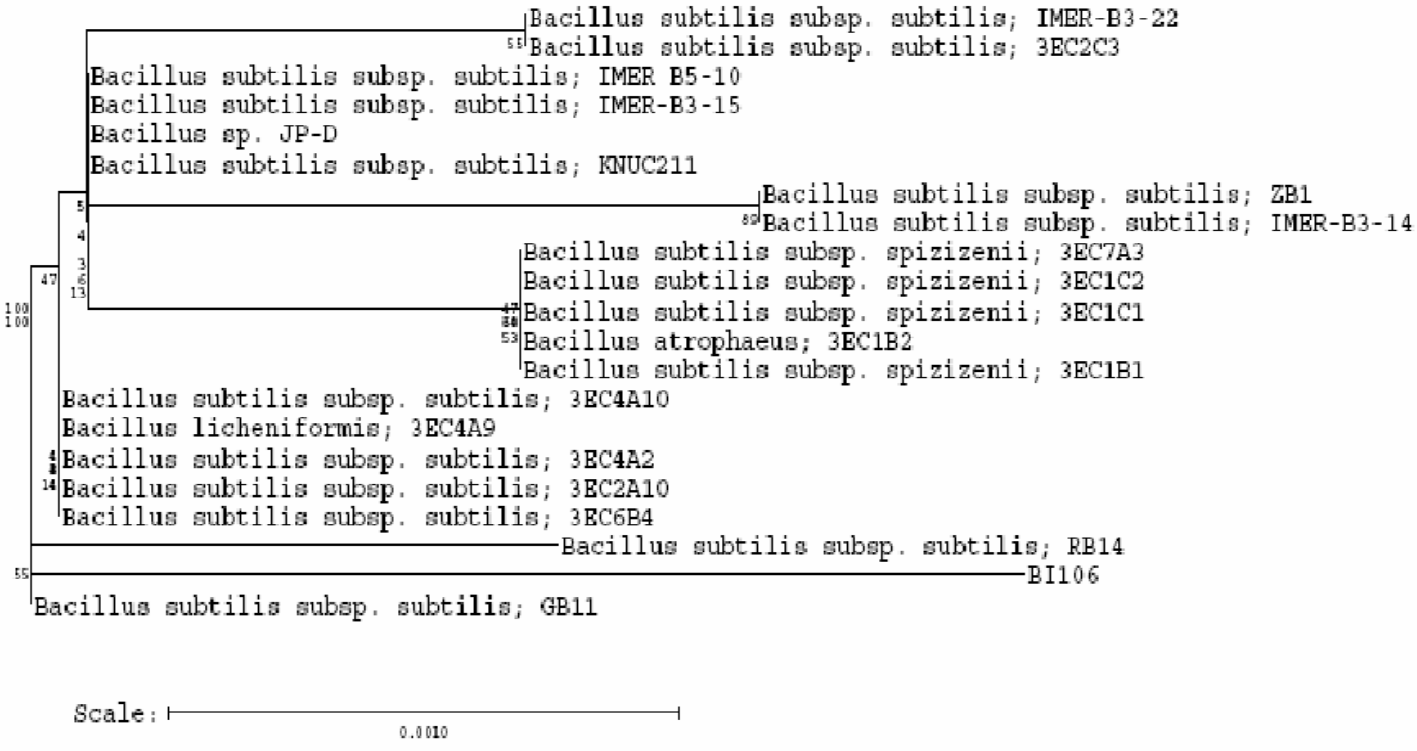


The amplified PCR product is shown in Fig.2. The 16s rRNA sequencing revealed that the organism was 0.999 related to *Bacillus subtilis subsp. subtilis GB11*; It was proved that it is a unique strain of *B. subtilis* with the ability to ferment lactose and produces *β*-galactosidase. The phylogenetic tree of the organism was constructed in accordance with previous reports (Weisburg *et al.,* 1991) (Fig.3). The probiotic spore forming *Bacilli* was tested for its antibiotic susceptibility with antibiotic discs and results are tabulated in Table 1.

Discussion

The isolation of a spore forming probiotic *Bacilli* sp. was successful which had extraordinary abilities to ferment lactose. The biochemical tests were not decisive in defining the exact strain of microorganism as no previous reports of a spore forming *Bacilli* sp. with a lactose fermenting ability was reported. The 16SrRNA sequencing revealed the strain to be *Bacillus subtlis* closely related to *Bacillus subtilis subsp. subtilis GB11*, which is the first of its kind to be ever reported to have lactose fermenting ability at room temperature. Previous reported strain *Bacillus subtilis KL88* (Khalid *et al.,* 1991) has a similar ability to ferment lactose at temperatures ranging between 0-10°C. We acclaim this special ability of fermenting lactose at room temperature by *B. subtilis* to its source of isolation dairy effluent. Over a period of time

Fig. 3. The phylogenetic tree of Bacillus subtilis



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this *B. subtilis* strain would have evolved to metabolize lactose. The sequencing data of this new strain has been submitted to National Centre for Biotechnology Information (NCBI), Europian Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ) and accepted for as new strain of *Bacillus subtilis SK09* NCBI-Genbank No:HM117721 (Sreekumar *et al.,* 2010). This particular strain of *Bacillus subtilis SK09* can be used as a probiotic supplement in lactose intolerant people. The antibiotic susceptibility of this *B. subtilis SK09* is of a very wide range and sensitive to most of the common antibiotics which makes it very safe for the use as a probiotic in human therapy for lactose intolerance.

Table 1. Antibiotic susceptibility of Probiotic spore forming Bacilli

Antibiotic (µg)	Diameter of inhibition (mm)
Ampicillin (10)	22 ± 1
Gentamycin (10)	25 ± 3
Streptomycin (10)	21 ± 3
Erythromycin (15)	26 ± 0.5
Clindamycin (2)	20 ± 1.5
Tetracyclin (30)	27 ± 0.5
Chloramphenicol (30)	18 ± 2
Ciprofloxacin (5)	33 ± 0.5
Amoxicillin (30)	19 ± 1
Methicillin (5)	24 ± 3

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