

Original Research Article

Identification and Characterization of *Enterococcus faecium* (MCC-2729), with Antimicrobial and Abiotic Stress Tolerance Properties

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ABSTRACT

Keywords

Enterococcus faecium,
Antimicrobial activity,
API50 CHL Test kit,
MALDI-TOF/MS,
16SrRNA

Thirty two isolates were obtained from soil and dairy samples of different regions of Andhra Pradesh and Tamilnadu. Among 32 isolates obtained only 10 isolates were confirmed as gram positive and catalase negative, which are the characteristic features of the lactic acid bacteria and these 10 isolates were further screened for antibacterial activity. Isolate CST-1 strain has shown higher zone of inhibition compared to the other isolates against *B.subtilis*, *E.coli*, *P.aeruginosa* and *S.aureus* when screened for antibacterial activity. The cells of CST-1 were round, non-motile and non-spore forming. This isolate was observed to grow optimally at 37°C and pH 6.0, it could also grow in media containing 2 - 6% (w/v) sodium chloride (NaCl). The antibacterial property of supernatant was also observed from the CST-1 grown in different conditions of pH, temperature and NaCl. Phylogenetic analysis based on 16S rRNA gene sequence and MALDI-TOF/MS methodology has confirmed CST-1 as *Enterococcus faecium*. On the basis of morphological, biochemical and phylogenetic studies it was confirmed as *Enterococcus faecium* and deposited in Microbial Culture Collection (MCC), Pune, India with an accession number of MCC-2729. 16S rRNA sequence was deposited in Genbank, EMBL with an accession number of LN713948.1

Introduction

Microbes are characterized by their extraordinary diversity in shape, size, and physiology. It is essential to classify them into groups based on their similarities and differences. Soil bacteria are responsible for the production of various biochemical products including majority of clinically useful antibiotics (Anita et al., 2014). Antibiotics are one of the important pillars of modern medicines (Ball et al., 2004), but

old antibiotics lose their efficacy and they are not replaced with equal number of new molecules (Hancock, 2007; Coates & Hu, 2007). Hence Considerable research is being done in order to find new antimicrobial metabolites producing bacteria (Courtis et al., 2003). It is established that proper identification and characterization of microorganisms is very important because it also broadens the scope for exploration of

many important microbial products. Infectious diseases are highly destructive to the social lives and very limited antibiotics are available to treat infections (Nikaido, 1994). To overcome these problems, there is a great need for new antibiotics, which can be used to treat various microbial infections. Hence, the development of new drugs with broad spectral activity, without side effects and better activity for treatment are required to fight effectively against infectious diseases (Demain and Sanchez, 2009).

Lactic acid bacteria (LAB) is known as most important non pathogenic bacteria that play a key role in producing vitamins, preservation of foods, and also protects mankind from various diseases due to their antimicrobial activity. These bacteria are also known from centuries for their importance in food preservation where the metabolites released from them play a major role. In recent years the use of Lactic acid bacteria as "Probiotics" is gaining more and more importance (Berg, 1996; Oberg *et al.*, 1998).

The organisms growing under different physical, biological and chemical conditions have exerted a driving force on their selection leading to new adaptive strategies and synthesis of new metabolites (Valentine, 2007). Hence, we tried to isolate lactic acid bacteria from unique sources, so that there is high chance of isolating bacteria that can produce novel metabolites. The present investigation deals with the characterization of bacteria, CST-1 strain which shows maximum antimicrobial activity under different stress conditions.

Materials and Methods

The chemicals and solvents used are analytical grade in the present study and were procured from Himedia, Mumbai. Test

organisms namely, *Bacillus subtilis* (MTCC-10403), *Staphylococcus aureus* (MTCC-3160), *Escherichia coli* (MTCC-1652) and *Pseudomonas aeruginosa* (MTCC-4676) were procured from IMTECH, Chandigarh, India. API50CHL test kit was procured from Biomerieux, France.

Isolation of Lactic acid bacteria Strains

Different dairy products and soil samples from milk remnants dumpyard for over 20 years were collected and serially diluted from 10^{-1} to 10^{-7} and plated on sterile de Man Rogosa (MRS) agar containing an enzymatic digest of animal tissue (10 g L^{-1}), beef extract (10 g L^{-1}), yeast extract (10 g L^{-1}), dextrose (20 g L^{-1}), sodium acetate (5 g L^{-1}), potassium phosphate (1 g L^{-1}), ammonium citrate (2 g L^{-1}), magnesium sulfate (0.1 g L^{-1}), and manganese sulfate (0.05 g L^{-1}). The plates were incubated at 37°C for 48 hr. The isolated pure cultures were labeled and stored as stock cultures at -80°C until further examination.

Morphological characterization of bacteria

The 24 h lactic acid bacteria culture was inoculated on MRS agar medium and incubated at 37°C for 48 h. After incubation colony growth pattern was studied. The morphological characteristics were noted by observation with a microscope and also after staining isolate was identified up to the genera level by comparing the morphology as described in Bergey's Manual of Systematic Bacteriology. Gram staining was done according to (Grams, 1884). Motility was observed by hanging drop method using 18 h bacteria CST-1 STRAIN culture (Craigie, 1931). Endospore formation was determined by malachite green staining (Salle, 1948).

Screening of Lactic acid bacteria Strains for Antimicrobial Activity

The isolated strains were screen for antimicrobial activity by using agar well diffusion method (Murray et al., 1995) on two gram positive and two gram negative bacteria namely *Bacillus subtilis* (MTCC-10403), *Staphylococcus aureus* (MTCC-3160), *Escherichia coli* (MTCC-1652) and *Pseudomonas aeruginosa* (MTCC-4676). The plates were incubated for 72 h at 37°C under aerobic conditions and the clear zones of inhibition were measured. The experiments were performed in triplicate, and the strains with antimicrobial properties were further characterized.

Scanning electron microscopy

The SEM studies were carried out by Danilatos, 1988. The bacteria CST-1 strain culture from 24 h nutrient agar plate was taken and fixed for 2 hr in 2% formalin. After washing with saline solution, the culture was dehydrated in 20-100% ethanol water series. The air-dried bacteria was coated with thin layer of platinum in Gatan cryostage (Hitachi S-900 FESEM) and scanning electron microscopy (SEM) was performed using ZEISS electron microscope at 20 kV accelerating voltage.

Biochemical Characterization of CST-1 strain

Different biochemical tests namely Indole test (Smibert et al.,1994), MR-VP test (Harden, 1906), Simmons citrate (Claus 1989), Starch hydrolysis (Bird & Hopkins 1954), H₂S production (Artman 1956), Catalase (Doelle & Editor 1969), Oxidase (Gordon & Mcleod 1928), Pyruvate fermentation (Steinkraus et al., 1969) Urease (Smibert et al.,1994), Nitrate reduction test (Skerman 1967), Gelatin

hydrolysis (Clarke & Cowan,1953) test were performed.

Identification of strain CST-1 by API 50CHL kit

The strains which had previously been isolated by pure culture were identified with an API 50 CHL Carbohydrate Test Kit (BioMerieux Co., France). These tests were conducted according to the instructions of the manufacturer, and by the database provided by Biomerieux (Hyun-jue Kim, 2006). The carbohydrate fermentation was performed following the standard method.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

The sample preparation for mass spectrometry was carried out according to (Thiago et al.,2013). Each steel slide contained three acquisitions groups, and each acquisition group contained 16 spots, being able to perform 48 different isolates. An amount of freshly grown 24-hour-old colony was placed directly onto a steel target sample spot in a thin film. The film was then overlaid with 1µl of a saturate matrix solution of α -cyano-4-hydroxycinnamic acid and dried at room temperature. The slide was then inserted into the MALDI-TOF MS instrument (BioMeri  ux, Marcy I'Etoile, France). The mass spectra generated were analyzed and compared with a reference spectra database.

Identification of bacterial Strain by 16S rRNA

Genomic DNA of the bacteria CST-1 STRAIN was isolated as described by Sam brook et al., 1989. Gene specific for 16S rRNA coding regions were amplified by PCR (Kawasaki et al., 1993) using universal

primers, forward (5'-AGTTTGA TCCTGGCTCAG-3') and reverse (5'-GGCT/TACCTTGTTACGACTT-3'). The numbering of positions in the 16S rRNA gene fragments was based on the E.coli numbering system (Borsius, et al., 1981). Amplified 16S rRNA gene products were purified by standard protocol and sequenced with an ABI PRISM big dye terminator cycle sequencing Ready Reaction kit on an ABI PRISM model 310 genetic analyzer. The 16S rRNA sequence of bacteria CST-1 STRAIN was deposited in the EMBL/GenBank/DBJ databases and compared with close relatives using the BLAST search tool (Thompson et al., 1994; Altschuf et al., 1997). The distance matrices of the aligned sequences were calculated by using the two parameters method of Kimura, (1980). The maximum likelihood method was used for constructing a phylogenetic tree.

Effect of NaCl, pH and temperature on bacterial growth and antimicrobial activity

Effect of NaCl

The MRS media is supplemented with 0-12% NaCl individually and the freshly grown bacteria is inoculated. The culture is grown at $37\pm 1^{\circ}\text{C}$ and the data was collected after 72hrs. The supernatant from bacteria grown for 72 hrs at different NaCl concentrations was collected separately by centrifugation and screened for antibacterial activity by agar well diffusion method on the above mentioned test organisms.

Effect of pH

The MRS media is adjusted to pH of 3-10 individually using 0.1N HCl or NaOH. The freshly grown bacteria is inoculated and the culture is grown at $37\pm 1^{\circ}\text{C}$. The data was

collected after 72hrs. The supernatant from bacteria grown for 72 hrs at different pH conditions was collected separately by centrifugation and screened for antibacterial activity by agar well diffusion method on the above mentioned test organisms.

Effect of temperature

The freshly grown bacteria is inoculated into different MRS media broth tubes and were grown at temperatures ranging from $4-60^{\circ}\text{C}$, individually and the data was collected after 72hrs. The supernatant from bacteria grown for 72 hrs at different temperatures was collected separately by centrifugation and screened for antibacterial activity by agar well diffusion method on the above mentioned test organisms.

Statistical analysis

All the studies were performed in triplicates. The results are presented as Mean \pm Standard deviation. The data represented was a mean of three replicates \pm SE [$P\leq 0.05$ (dmrt)]

Results and Discussion

A total of 32 representative colonies were picked up and purified by performing repeated plating technique on MRS agar medium. Among 32 isolates obtained, only 10 putative isolates were gram positive and catalase negative, characteristic feature of the lactic acid bacteria. The potent isolates were further screened for antimicrobial activity using agar well diffusion method. However out of the 10 isolates, the isolate CST-1 exhibited maximum zone of inhibition, when compared to other isolates (Figure 1). The zone of inhibitions of different isolates on test organisms viz *E.coli*, *P.aeruginosa*, *B.subtilis*, *S.aureus* are given in Table 1.

Among the 10 lactic acid bacteria isolates, the isolate CST-1 has shown maximum zone of inhibition, thus the isolate CST-1 was subjected to further studies.

The morphology of the CST-1 strain was small, circular, moist, opaque and white colour round colonies, gram positive, non motile and non spore forming. The size of the cocci is between 748.4nm to 763.8nm as per the scanning electron microscope studies (Figure 2).

The isolate CST-1 was further subjected to biochemical characterization and the results were further illustrated in (Table 2).

The credibility of the results was rechecked by API50CHL kit and the test results reveal that the strains CST-1 is *Enterococcus faecium* (Table 3). The other isolates namely TSS-1, TSS-2, TSS-3, CCD-1, CCD-2, CST-2, CST-3, CST-4, and CST-5 were found to be *Lactobacillus brevis*, *Pediococcus damnosus*, *Lactococcus lactis*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus casei*, *Enterococcus faecalis*, *Sataphylococcus simulans* respectively.

Molecular confirmation of the bacteria

MALDI-TOF/ MS results have further confirmed that the strain CST-1 as *Enterococcus faecium*. The confidence value established by this technique for *Enterococcus faecium* CST-1 isolate was 98.6 (Figure 4).

The 16S rRNA of *Enterococcus faeci etalum* when sequenced generated 1200 bp long sequence (Figure 5) and the sequence was deposited in Gene Bank with an accession number LN713948.1. The homology studies have shown that 16S rRNA sequence exhibits more than 99% similarity with the *Enterococcus faecium* (ATCC-19434^T)

compared to other closely related *Enterococcus* sp. (Figure 6). From the phylogenetic tree, it was depicted that *Enterococcus faecium* is closely related to *Enterococcus lactis* and *Enterococcus ratti* which are not studied till date for their antimicrobial compounds. The isolated *Enterococcus Faecium* was deposited in Microbial culture collection, Pune, India and the issued accession number is MCC-2729.

Impact of abiotic conditions on the growth of *Enterococcus faecium*

The *Enterococcus faecium* was observed to grow in a temperature range of 25°C to 45°C with an optimum growth around 37°C. A range of acidic and basic conditions were tolerated by the bacteria. The optimum growth was observed between pH of 6 and 7 shown in Graph 1. The *Enterococcus faecium* even though isolated from milk dumpyard exhibited tolerance to different levels of NaCl. The optimum growth occurred between salinity of 2-4% (NaCl, w/v) and upto 6% NaCl (w/v) is tolerated. Bacterial metabolism is sensitive to salt, because salt exhibits specific ionic water binding properties (Korkeala et al., 1992). A similar growth result was observed with *Lactobacillus amylovorus* DCE-471 (Patricia et al., 2003). The physiological characterization of the *Enterococcus faecium* strain is shown in the (Table 3).

Enterococcus faecium might have acclimatized to soil environment with high osmotic concentration, wide pH range as degraded milk products are regularly dumped in the area. The high antimicrobial activity of the *Enterococcus faecium* allows it to survive the competition in its highly nutrient natural habitat and it is also a promising feature to carry out further studies in the isolation and characterization of the antimicrobial compounds from it.

Table.1 Zone of Inhibition shown by isolated species on different test organisms

Isolated colonies	<i>Bacillus subtilis</i> (MTCC-10403)mm	<i>Staphylococcus aureus</i> (MTCC-3160)mm	<i>Escherichia coli</i> (MTCC-1652)mm	<i>Pseudomonas aeruginosa</i> (MTCC-4676)mm
TSS-1 (Isolate-1)	4±0.2	4±0.2	5±0.1	4±0.2
TSS-2(Isolate-2)	6±0.2	4±0.2	3±0.1	3±0.2
TSS-3 (Isolate-3)	7±0.4	3±0.4	4±0.2	5±0.3
CCD-1(Isolate-4)	5 ±0.4	4±0.3	2±0.1	5±0.2
CCD-2(Isolate-5)	3±0.3	3±0.5	4±0.2	2±0.3
CST-1(Isolate-6)	10±0.4	8±0.2	4±0.1	5±0.3
CST-2 (Isolate-7)	7±0.1	3±0.2	3±0.2	5±0.4
CST-3(Isolate-8)	8±0.5	6±0.2	4±0.3	4±0.1
CST-4 (Isolate-9)	8±0.1	4±0.2	3±0.4	5±0.2
CST-5(Isolate-10)	4±0.3	6±0.2	3±0.4	5±0.5

Table.2 Biochemical Parameters of bacteria strain CST-1

Biochemical test	Result
Indole Test	Negative
Methyl Red Test	Negative
Voges Proskauer Test	Negative
Growth On Mac-Conkey Agar	Positive
Citrate Utilization Test	Positive
Arginine Dihydrolase	Negative
Casein Hydrolysis	Positive
Catalase Test	Negative
Cytochrome Oxidase	Positive
Gelatin Hydrolysis	Positive
H ₂ S Production	Negative
Lysine Decarboxylase	Negative
Nitrate Reduction	Positive
Oxidation/Fermentation	Oxidative
Starch Hydrolysis	Negative
Urea Hydrolysis	Negative

Table.3 Impact of physical parameters on growth of CST-1

Temperature (°C)	Growth	pH	Growth	NaCl (%)	Growth
4	—	3.0	+	0	+
10	—	4.0	+	1.0	+++
25	+	5.0	+	2.0	+++
30	++	6.0	+++	4.0	+++
37	+++	7.0	++	6.0	++
45	+	8.0	+	8.0	—
60	—	9.0	—	10.0	—
		10.0	—	12.0	—

+ Low growth; ++ Medium growth; +++ High growth; - No growth

Figure.1 Zone of Inhibition shown by 5 isolates against *B.subtilis* viz., 1: CST-1, 2: CST-5, 3: CST-14, 4: TSS-15, 5: CST-9

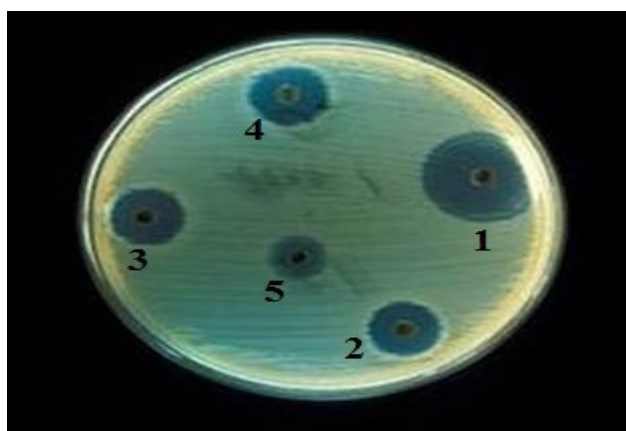
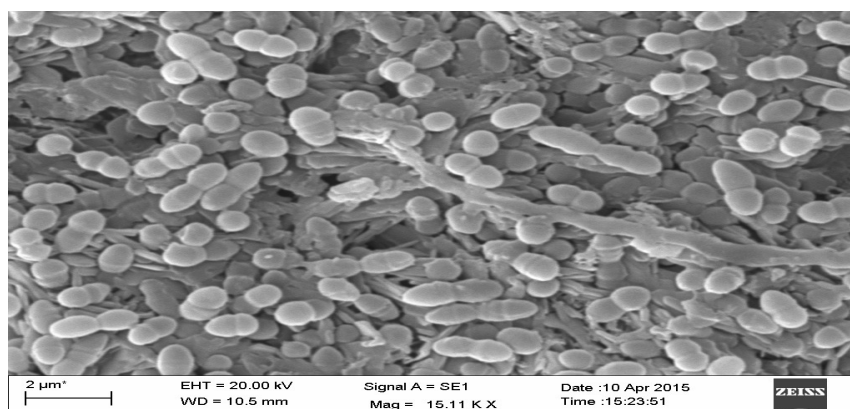


Figure.2 Scanning electron Micrograph of CST-1



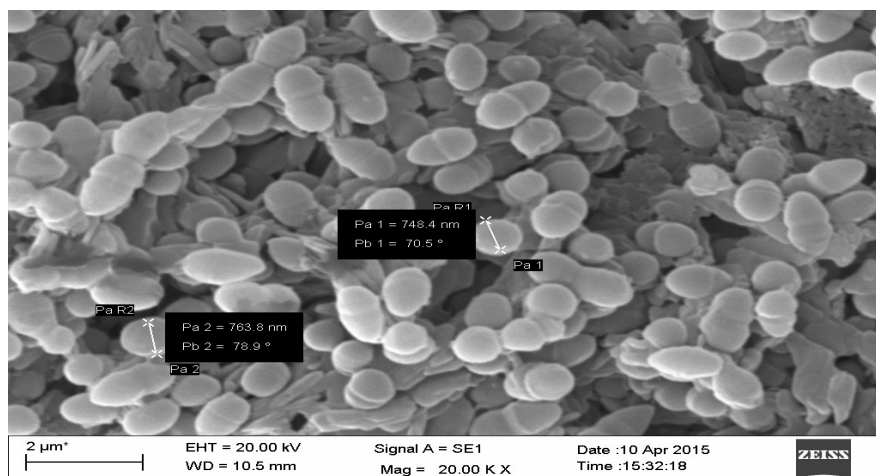
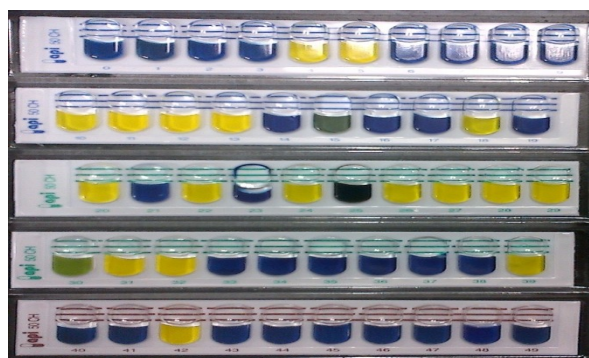


Figure.3 API 50 CHL strip analysis for strain CST-1



Yellow indicates positive; Blue indicates negative; Green indicates positive/negative
Black indicates positive for esculin test

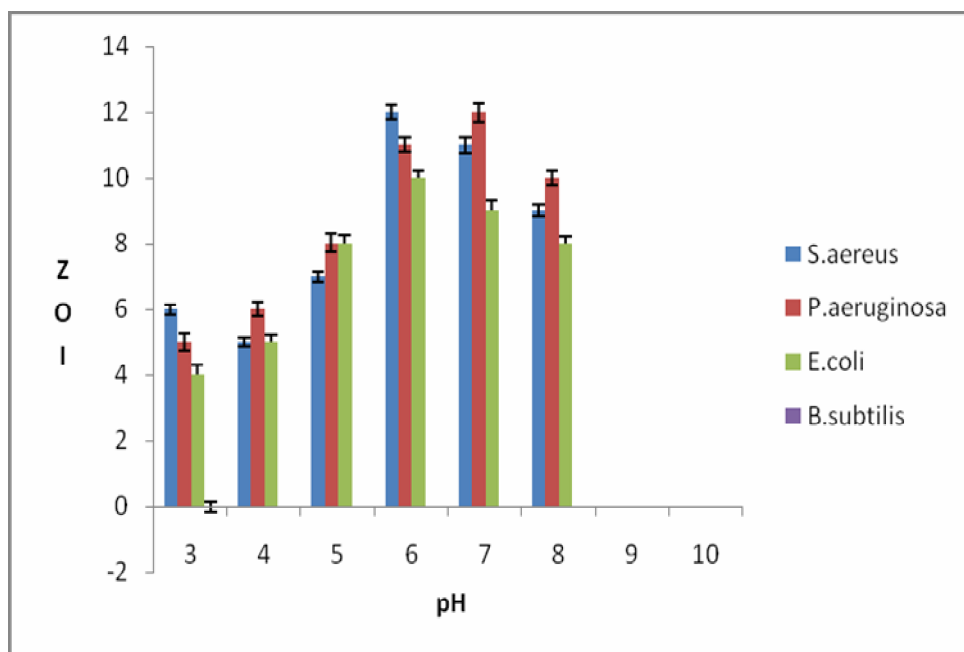
Figure.4 Identification of strain CST-1 by MALDI-TOF

VITEK® MS Results Approved	
Accession ID	ARD04-1
Organism Name	Enterococcus faecium
Organism Type	Protocol Bacteria
Confidence Value	98.6
Confidence Level	Medium
User Consolidated	No
Computation Engine	MS-CE IND_1.0.0
Review Status	Approved
Setup Operator	Prathyusha
Setup Date	29/08/14
Bench name	PREPSTATION1
Slide ID	DS130916079
Position	B4
Instrument	VITEKMSACQ01
Selection Operator	Prathyusha
Selection Date	2014082912309
Review Operator	Prathyusha
Review Date	29/08/14
Review Operator E-signature	Prathyusha Argula
Approval Operator	Shankar
Approval Date	29/08/14
Approval E-signature	Shankar Pasupuleti
Comment	Commented by : Shankar AR&D Isolate

Figure.5 The 16S rRNA gene sequence of bacteria strain *Enterococcus faecium*

TGGAACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGG
CGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTG
GGACTGAGACACGGCCCAAACCTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAA
TGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTA
AAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTTCATCCCTTGACGGTA
TCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGACGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTCTTAAGTC
TGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGG
AGGAACACCAGTGGCGAAAGGCGGCTCTCTGGTCTGTAAGTACGCTGAGGCTCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACT
CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTGCAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTATTGTTAGTTGCCATCATTAGTTGGGCACTCTAGCAAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACC
TGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTA
AGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCACGCTGCAACTCGCCTGCATG
AAGCCGGAATCGCTAGTAATCGC

Figure.6 Phylogenetic relationship of strain CST-1 and *Enterococcus* sp. based on 16S rRNA gene sequence



The data represented was a mean of three replicates \pm SE [$P \leq 0.05$ (dmrt)]

Figure.7 Effect of pH on antimicrobial property of supernatant and its zone of inhibition (ZOI)

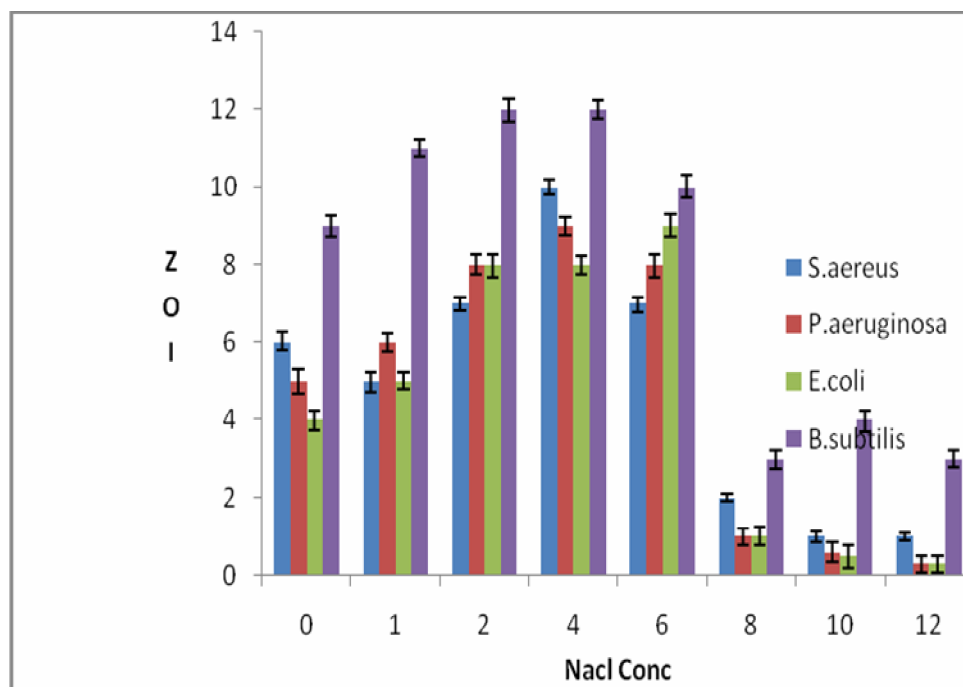
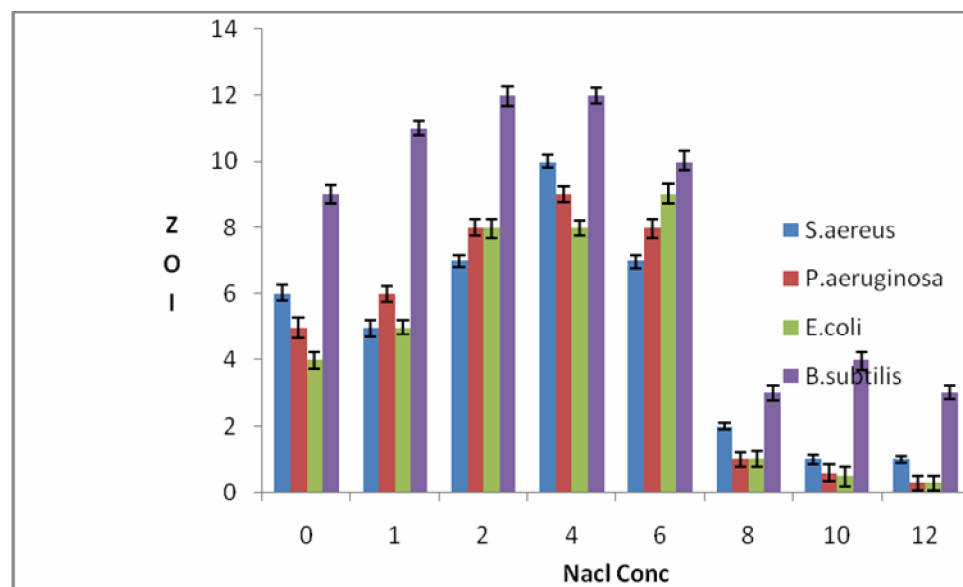
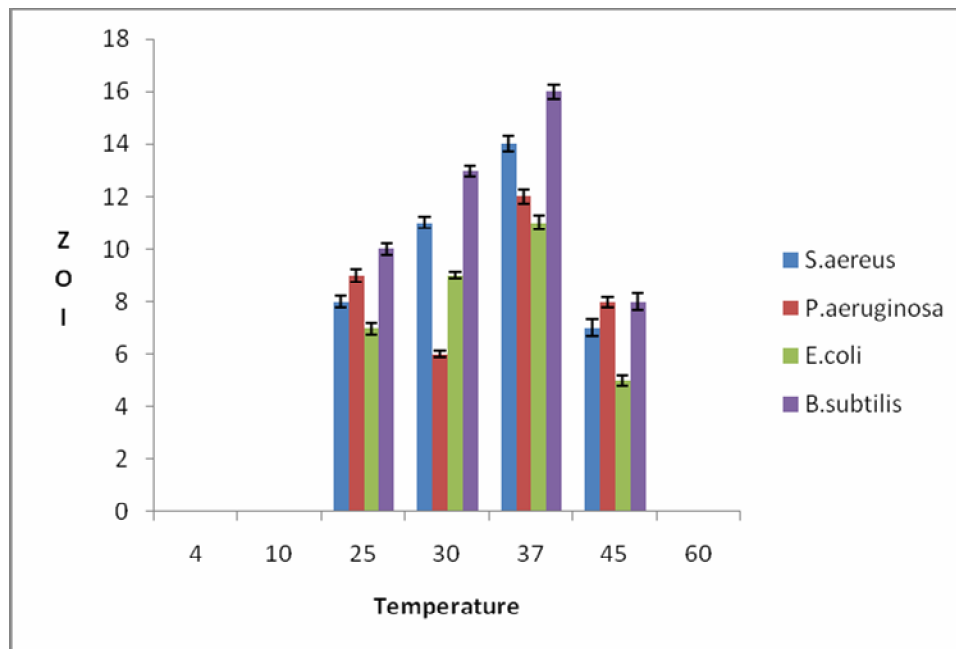


Figure.8 Effect of NaCl on antimicrobial property of supernatant and its zone of inhibition(ZOI)



The data represented was a mean of three replicates \pm SE [$P \leq 0.05$ (dmrt)]

Figure.9 Effect of Temperature on antimicrobial property of supernatant and its zone of inhibition (ZOI)



The data represented was a mean of three replicates \pm SE [$P \leq 0.05$ (dmrt)]

The growth parameters discussed above also justify that the isolated organism belongs to *Lactobacillus* family as it sustained growth between 4 to 45°C, upto 6.0% NaCl, survival at extreme conditions pH upto 8 (Teixeira et al.,2007).

Impact of abiotic conditions on the antimicrobial activity of *Enterococcus faecium*

A large number of factors influence the growth and production of antimicrobial metabolites by *Enterococcus faecium*. Parameters like NaCl, temperature, pH etc have profound effect on production of antimicrobial metabolites. Together with these parameters, the combination of media components also influences growth and metabolite production.

The production of antimicrobial metabolites by *Enterococcus faecium* was optimal from pH 3-5, increased between pH6-7 and found

to decline from pH8-10 which was illustrated in (Figure 7). Sodium chloride is shown to possess a marked effect on antimicrobial activity by *Enterococcus faecium*. In our study, increase in salt concentration from 0 to 4 % concentration showed an increase in antimicrobial activity thus proving that under salt stress the antimicrobial products are further induced. Beyond 6% concentration of salt, there is no growth of *Enterococcus faecium* but the reported activity might be of NaCl which is known to have antimicrobial activity (Figure 8).The incubation temperature has also shown major impact on antimicrobial activity. The optimum temperature ranges for *Enterococcus faecium* ranges from 25 to 45°C respectively. Incubation temperature of 37 °C was found to be optimum for maximum antimicrobial activity illustrated in (Figure 9).

On the basis of morphological, physiological, biochemical, molecular and

phylogenetic analysis, isolate CST-1 belongs to genus *Enterococcus*. The isolate showed closest similarity to the species *faecium*. It has been deposited in the Microbial Culture Collection, Pune, India as *Enterococcus faecium* with deposition number MCC 2729. The 16S rRNA gene sequence is deposited in Gene Bank (EMBL), with a Gene Bank accession No. LN713948.1 was allotted. The bacterium was found to tolerate different conditions like pH, temperature and NaCl and also produced antimicrobial compounds under these parameters. Hence, *Enterococcus faecium* (MCC - 2729) might be a potential source of antimicrobial compounds which can be used in therapeutics or probiotics.

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