Characteristic of Probiotic Enterococci isolated from Basi Bhat

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Abstract::
Enterococci sp. is isolated from Basi Bhat. It had aggregation time (62 min) and antibacterial effects against Proteus vulgaris. It showed protease activity while amylase and lipase activity absent. It passes cell surface hydrophobicity-83.2% ; resistance to acidic condition (pH 3 for 90 min) and growing in presence of bile salts (in culture medium containing more than 0.15% bile salt). It had resistance to amphotericin, cefpodoxime, cloxacillin, cephalothin, flucanazole, ketoconazole, oxacillin, and vancomycin. This research showed Enterococci sp. is appropriate for probiotic use.

Key words: *Enterococci, probiotic, Basi Bhat, autoaggregation, coaggregation.*

Introduction:

Probiotics are defined as live microbial food supplements that beneficially affect the host by improving intestinal microbial balance. Among the potential probiotics, *Enterococci* can be used to reduce diarrhea in chicken, pig, cattle, etc.*Enterococci* are Gram-positive cocci, and classified as Kingdom: *Bacteria*, Phylum: *Fimicutes*, Class: *Bacilli*, Order: *Lactobacillales*, Family: *Enterococcaceae*, Genus: *Enterococcus*. It can survive in harsh conditions in nature and found in soil, water, and plants. Some strains are applied in the manufacture of foods while others pathogenic to human and animal (e.g. colonize the gastrointestinal and genital tract). *Enterococci* can grow at 10 to 42°C and in broad pH range. *Enterococci* form short chains or are arranged in pairs, even may appear cocobacilli. In general,*Enterococci* are □-hemolytic. *Enterococci* are typically catalase negative, and are facultative anaerobic. They are able to grow in 6.5% NaCl, also resist 3.5 % bile salts. *Enterococci* are resistant to many antimicrobial drugs, "including cell-wall active agents; aminoglycosides, penicillin and ampicillin, and vancomycin". *Enterococcal* isolates with lowered susceptibility to vancomycin are categorized as vanA, vanB, and vanC. While VanA and vanB cause the greatest threat as, carrying resistance genes on a plasmid. Thus plasmid carrying resistance genes are transferable, by conjugation. Enterococcal strains that are vancomycin-dependent have been found, but are rare and less common than vancomycin-resistant strains (referred to as "VRE") (Arias et al., 2007).

They are capable of not only fermentation to produce lactic acid but also can "catabolize various energy sources from carbohydrates, glycerol, lactate, malate, citrate, diamino acids and many α-keto acids". Sugars metabolized by them include: D-glucose, D-fructose, lactose, maltose (Macovei et al., 2006).

Material and methods:

Isolation of Enterococci:

The Enterococci sp. was isolated from Basi Bhat a conventional and tribal food of India on medium containing; rice powder: 0.5%, peptone: 0.5%, K_2HPO_4: 0.2%, $MgSO_4$: 0.05%, $FeCl_3$: traces, and agar: 2%.

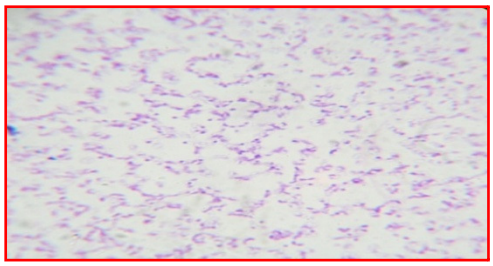


Figure 2: Coaggregation of Enterococci with elongating yeast in natural isolate

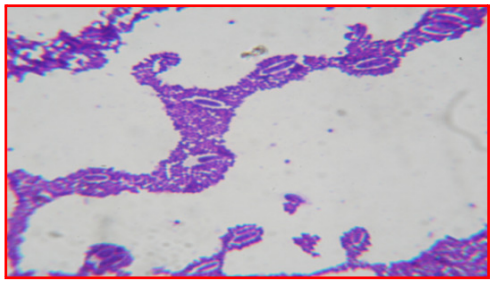


Figure 1: Diplococcal arrangement of Enterococci

Detection of antagonistic activity:

For detection of inhibitory activity, the well diffusion assay was used. Plates containing solidified nutrient agar (10 ml) overlaid with 10 ml of soft nutrient agar (0.7% agar in nutrient broth) were inoculated with 5 □l of an overnight culture of *S. aureus, S. typhi, Proteus vulgaris or E. coli.* Wells were cut in the agar and 30□l of the culture supernatant of *Enterococci* isolate transferred into the well. The plates were incubated aerobically for 24 h at 37°C, and examined for clear inhibition zones around the wells. The same was repeated twice.

Aggregation test:

Aggregation was considered positive when bacteria gravitated to the bottom of the tubes, leaving a clear supernatant fluid. The test was examined every 15 min for 2 h.

Table 1. Different attributes of *Enterococci sp.*

Parameter	
Amylase activity	-
Protease activity	+
Lipase activity	-
Aggregation time (min)	62
Cell surface hydrophobicity (%)	83.2

Cell surface hydrophobicity test:

It was determined by the method of Rosenberg et al. (1980). The strain was harvested after 18 h of growth, washed twice and suspended in saline solution to an optical density (OD) of 0.5 at 600 nm. To 3 ml of washed cells, 1 ml of toluene was added. The mixtures were blended for 90 seconds. The tube was left to stand for 15 min for separation of the two phases and the OD of the aqueous phase was taken. Hydrophobicity was given by the percentage decrease in the OD of the bacterial suspension due to partitioning of cells into the hydrocarbon layer. Percentage of hydrophobicity = [(OD600 before mixing - OD600 after mixing) / OD600 before mixing] x100.

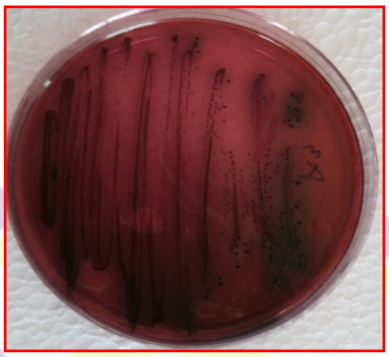
Co-aggregation test:

Reid et al. (1988) suggested the ability of probiotic organisms to interact closely with pathogens such as *E. coli* would constitute an important host defense mechanism against infection. Thus co-aggregation was investigated according to Jin et al. (1998). Suspension of the strain, *E. coli, S.aureus,Proteus vulgaris*, or *S.* typhi was adjusted in phosphate buffer (pH 7) to an OD600 of 0.5. A suspension (0.5 ml) of each pathogen and a similar suspension (0.5 ml) of isolated strain was placed

together in a test tube and mixed thoroughly. The OD600 of the bacterial mixture was measured after incubation for 4 h at 37°C. Control tubes contained 1 ml of a suspension of each bacterial species. The percentage of co-aggregation was given by the equation of Handly et al. (1987): Percentage of co-aggregation = {[(PC+LC)/2-(P+L)] / (PC+LC)/2} ×100 where PC and LC represent the optical densities in control tubes containing only pathogen or Enterococci after 4 h of incubation, respectively; P+L represent the optical density of the mixed culture after the same period of incubation.

Bile salts tolerance test:

It was determined by sub culturing isolated Enterococci strain on bile salt containg media like DCA, SS agar, and MacConkey agar aerobically at 37°C for 48 h.



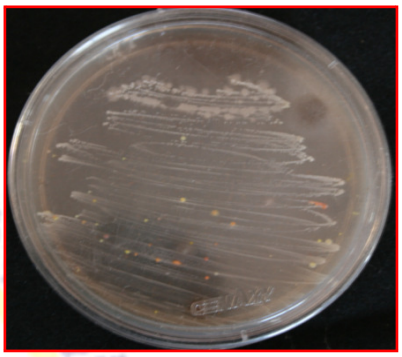


Fig.3: Growth on MacConkey **Fig.4:** Growth on DCA**Fig.5:** Chromogenic Bacteria

Detection of enzymatic activities:

For amylase activity, the strain cultured on nutrient agar containing2% starch. For detecting the clear zones of amylase activity, dense Lugol's solution was poured over the plates. In the case of lipase activity, nutrient agar containing olive oil (1%) and arabic gum (1%) was used to culture the strain. For detection of protease activity, the strain was cultured on nutrient agar medium consisting of 1% skim milk.

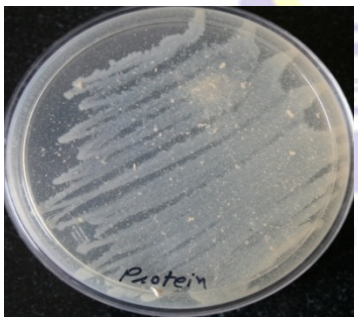


Fig. 6: Protease activity (+ve) **Fig. 7:** Lipase activity (-ve) **Fig. 8:** Amylase activity (-ve)

Acidic pH tolerance test:

Cell suspension was prepared as above and then diluted 1 x 10^{-5} in phosphate buffer at pH 3 and 6. The suspensions were then incubated for 90 min at 37°C. The viability were checked by growing the bacterial suspensions on aerobically at 37°C for 48 h (Garriga et al., 1998).

Antibiogram test:

The inoculum of Enterococci was spread evenly over the entire surface of the plates containing nutrient agar. Subsequently, paper discs containing the antibiotics were placed on the plates and incubated aerobically at 37°C for 24 h.

Table. 2-Antibiogram profile of *Enterococci*

Antibiotic	Abb	Halo zone (mm)	Antibiotic	Abb	Halo zone (mm)
Amikacin	AK^{10}	18	Flucanazole	Fu^{25}	-
Amoxycillin	AM^{30}	0.8	Kanamycin	K^{30}	1.6
Amphotericin	Ap^{100}	-	Ketoconazole	Kt^{10}	-
Ampicillin	AMP^{10}	+	Nalidixic acid	Na^{30}	+
Carbenicillin	CB^{100}	1.6	Nystatin	Ns^{100}	-
Cefpodoxime	Cep^{10}	-	Neomycin	N^{30}	1.8
Ceftazidime	CA^{30}	1.2	Ofloxin	Of^{5}	2.1
Ceftriaxone	Ci^{30}	1.3	Oxacillin	Ox^{5}	-
Chloramphenicol	C^{30}	1.3	Piperacillin	Pc^{100}	1.4
Chlorotetracyclin	Ct^{30}	1.2	Rifampicin	R^{30}	1.1
Clarithromycin	Cw^{15}	+	Roxithromycin	Ro^{30}	+
Cloxacillin	Cx^{30}	-	Streptomycin	S^{10}	1.0
Cephalothin	CH^{30}	-	Tobramycin	TB^{30}	0.9
Imipenem	I^{10}	2.4	Vancomycin	VA^{30}	-

Results and discussion:

From Basi Bhat total 19 Enterococci were screened based on aggregation time, antibacterial effects, enzymatic activity, cell surface hydrophobicity, co-aggregation, tolerance to bile salts and acidic condition and finally selected Enterococci DI4 as a source of probiotic because of its predominant characteristics in comparison to the other isolated strains from the Basi Bhat. The results showed that *Enterococci* had no amylase and lipase activity; however, it showed protease activity. *Enterococci* had antibacterial effect against *Proteus vulgaris* but not against *E.coli, S.aureus,* and *S.typhi*. Also it had aggregation time - 62 min and cell surface hydrophobicity - 83.2%. Aggregation shows clumping of strains together and also adhesion ability to the epithelial cells indirectly but in a strong way. It has been reported that bacteria which shows low aggregation time, also have high cell surface hydrophobicity and adhesion ability to the mucus (Taheri et al.,2009). Aggregation and cell surface hydrophobicity of the bacteria could be used instead of the examination of adhesion ability to mucus, because there is a strong relationship among these characteristics especially between aggregation time and adhesionability to the epithelium of the digestive tract (Garriga et al., 1998). The effects of bile salts on the survival of lactobacilli also have been investigated by several authors and are thought to be linked to the ability to de-conjugate bile acids (Tannock et al., 1997). *Enterococci* showed resistance in culture medium containing bile salts 0.15% in MacConkey, 0.5% in DCA, 0.55% in SS agar. Table 2 reveals that, there was not any growth inhibition against this strain in amphotericin, cefpodoxime, cloxacillin, cephalothin, flucanazole, ketoconazole, oxacillin, and vancomycinantibiotics. In general, *Enterococci sp*. had appropriate probiotic properties.

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