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Original Research Article

Probiotic Characteristics of Anti-Candida *Bacillus Tequilensis* Isolated From Sheep Milk and Buffalo Colostrums

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ABSTRACT

Recently, probiotics are highlighted for their ability to inhibit human pathogens. *Candida* is a common commensal and opportunistic pathogen of human being. The present study aims to isolate and characterize probiotic bacteria from various food samples and screen their ability to inhibit pathogenic *Candida* species. Among the various samples screened culture isolated from sheep milk (SHW) and Buffalo colostrums (COBT) showed anti-candida activity. These cultures also showed probiotic potential such as non-hemolytic on sheep blood agar, production of lactic acid as well as hydrogen peroxide, tolerance to range of pH 4-9, ox bile tolerance up to 2% and tolerance to spermicide. The isolates were identified as *Bacillus tequilensis* by 16sRNA sequencing and would be an ideal candidate for further probiotic characterization.

***Key Words*:** Probiotics *Candida* species, *Bacillus tequilensis*

INTRODUCTION

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host". [1] Many bacteria such as *Lactobacillus, Lactococcus, Bifidobacterium, Leuconostoc, Pediococcus, Bacillus* and many others are included in the list of probiotics. [2,3] Recently, probiotic micro-organisms have gained much popularity due to their ability to control various ailments including bowel disorders, allergies along with other benefits provided by them. [4,5] Moreover, there are reports of probiotic strain showing anti-microbial activity. [6,7] Due to this popularity, there is a huge increase in market share of probiotics. [8,9] *Candidiasis* is one of the most important nosocomial infections caused by opportunistic pathogen, *Candida*, affecting the skin, oral cavity, esophagus, gastrointestinal tract, vaginal area and vascular system. The excessive uses of anti-fungal agents have created many varieties of *Candida* that are resistant to commonly used anti-fungal drug. [10,11] Hence scientists worldwide are looking for an effective alternative measure for candidiasis. One of the preventive advances may be the use of probiotic bacterial species, which are commonly found in the traditional food preparations and are not hazards for the human beings.

Many bacteria are reported for their ability to inhibit Candida species, most important among them are bacillus species. More over there are reports on use of bacillus as probiotics. Much research with Bacillus has been performed in animals and some clinical studies in humans. This

species is a widely used oral vaccine delivery system since it has been categorized as a novel probiotic for both human being and animal consumption. [12,13] A probiotic with anti-candida activity would be highly beneficial since it can eliminate the use of antibiotics especially during pregnancy.

Considering these points the present work is aimed to isolate anti-*Candida* bacteria from various food sources and screen its probiotic potential.

MATERIALS AND METHODS

Sample Collection and Isolation of Bacteria:

Samples such as milk of Sheep, Goat and Buffalo, Buffalo colostrums, curd made from Cow and Buffalo milk, collected from various places in Sangli and Pune District, Maharashtra, India, were used for isolation of Probiotic cultures. Samples were collected in sterile tubes and stored at cool conditions during transport. All samples were inoculated in different media such as De Man, Rogosa and Sharpe agar (MRS), and Sabouraud Dextrose agar after serial dilution. MRS medium was incubated under anaerobic conditions. Cultures were incubated at room temperature for 48 hr. Isolated colonies with different morphologies were selected, streaked on agar slants and preserved for further use. These cultures were inoculated in the respective broth with 1%, 1 OD density and incubated for 48 and used for all experiments.

Screening of Anti-*Candida* Activity

Anti-candida activities of the isolates were screened by using agar well diffusion method against *Candida albicans* NCM 3557. *Candida* culture (100 µl, 1 OD at 600 nm) were platted on Sabouraud's dextrose agar and incubated for 30 min before making wells using agar well borer (4mm dia.). [14] Supernatant (20 µl) of 48 hr grown bacterial isolates were added in each well and incubated at 37^0C for 24 h. Fluconazole (20µl 1 mg/ml) was used as positive control. [15]

Thermal stability of anti-candida compound:

To study the thermo stability of the bioactive compound present in culture supernatant, aliquots of 5 ml of cell−free supernatant were incubated for 30 min at various temperatures ranging from 40°C to 100°C and 120°C (autoclaving 30 min). After the heat treatment, the samples were cooled to room temperature. Anti-candida activities of these heat treated samples were determined by agar well diffusion method. Untreated culture supernatant was used as control. [16]

Effect of proteolytic enzyme:

Effect of proteolytic enzyme on anti-candida activity was checked by treating culture free supernatant with proteinase K in phosphate buffer in 1:1 ratio. The mixture was incubated for 2 hrs at 37°C and proteinase K was inactivated by heating at 100°C for 3 min. The anti-candida activity of the mixture was then checked by agar well diffusion method. [17, 18]

Probiotic Characterization

Hemolytic Activity:

Blood hemolysis was assessed on MRS agar plates supplemented with 5% sheep blood. Each bacterial suspension was streaked on the blood agar plates, incubated at 37°C for 24 hrs and checked for α, β and γ-hemolysis pattern. [19]

pH Tolerance Test:

The isolated bacterial cultures were inoculated into sterile MRS broth tubes of varying pH (i.e. 3, 4, 5, 6, 7, 8 and 9). Tubes were incubated at 37°C for overnight and growth was checked by measuring optical density at 600nm. [20]

Bile Tolerance Test:

Bile tolerance test was performed as per Le et al. 2015, with modification. Accordingly, bacterial cultures were inoculated into 10 ml of MRS broth containing 0.5%, 1.0%, 1.5% and 2.0% (w/v) Ox-bile (Sigma–Aldrich) and total viable count was measured after incubated for 6 hr incubation at 37°C. The TVC was then compared with control. [21]

Bile Salt Hydrolase Assay:

Bacterial strains were tested for hydrolase activity (BSH) against tauro- or glyco-CBA by using a plate assay method. Bacterial cultures were streaked on MRS agar supplemented with taurodeoxycholic acid (0.5% wt/vol.) The plates were incubated for 48 h at 37°C. BSH activity was detected by observing the precipitation of deoxycholic acid on agar medium around colonies. [22]

Determination of Lactic Acid Production:

Lactic acid was determined by titration method by adding 0.1M NaOH into 25ml of the supernatant (collected after 120 hr of incubation) containing phenolphthalein as indicator. End point was the appearance of pink colour. Each ml of 0.1M NaOH is equivalent to 90.08mg of organic acid. [23]

Determination of Hydrogen Peroxide Production:

Hydrogen peroxide production was detected using titration using 0.1M potassium permanganate against 25ml of the supernatant containing 20ml of 0.1M H_2SO_4. Decolorization of the solution was regarded as the end point. Each ml of 0.1M potassium permanganate is equivalent to 1.079 mg of hydrogen peroxide. [23]

Spermicidal Susceptibility Test:

Spermicidal susceptibility test was performed using MRS medium containing 5%, 10%, 15% Nonoxinol-9, a commonly used spermicidal compound. Bacterial suspension 1 OD 1% were inoculated in the medium and incubated at 37^0C. Total viable count of the culture were measured at regular intervals and compared with control. [24]

Identification of Cultures

Bacterial cultures were identified by 16sRNA genes sequencing as per [25] and the sequence were compared with reference sequences available in GenBank using the BLAST algorithm. [26]

RESULTS

The present study was aimed to screen different food materials for anti-candida bacteria and to explore their probiotic potential. Total 29 cultures were isolated from various samples in Sabouraud dextrose agar under aerobic conditions. Out of which two cultures designated as SHW isolated from sheep milk and COBT isolated from Buffalo colostrums exhibited anti-candida activity (Fig.1). These two cultures were found to be Gram positive and spore forming cultures. The anti-candida activity of the culture was found to be in the culture supernatant. From MRS medium five different types of colonies were isolated under anaerobic conditions. However, these cultures failed inhibit *Candida.*

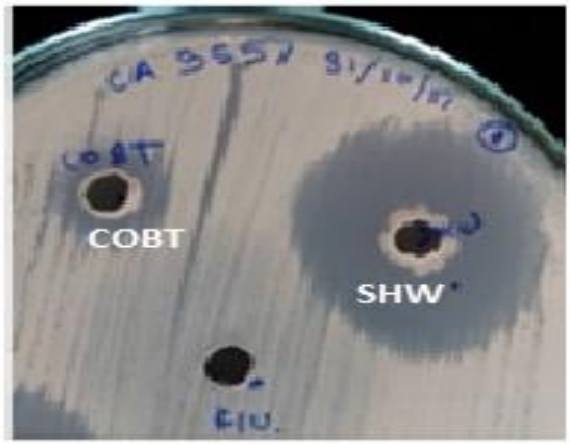

Fig.1- Screening of anti-candida activity of isolated *Bacillus tequilensis*.

Supernatant of both isolates were tested for temperature tolerance and found to be tolerant to range of temperature (40^0 to 120^0 C for 30 min) indicating the heat stability of the active molecule (table 1).

Table 1. Heat stability of *Bacillus tequilensis* against *Candida* albicans [zone of inhibition in cm]

Cultures [30 µl vol.]	CA 3557				
	control	40^0	60^0	100^0	120^0
COBT	2.8	2.8	2.7	2.2	2.0
SHW	3.2	3.2	3.0	3.0	2.2

Similarly, the cultures could tolerate to wide range of pH. The SHW tolerated pH 4 to 9 and COBT 5 to 9. Maximum growth of both cultures was observed at neutral pH and at low pH growth was found to be negligible. Supernatant of the COBT culture lost complete anti-candida activity when treated with proteolytic enzyme and Supernatant of the SHW culture was reduced anti-candida activity when treated with proteolytic enzyme Proteinase K. This

indicates that active molecule present in COBT is proteinaceous in nature. But in case of SHW the anti-candida activity is reduced. Thus, protease resistance ability of antifungal protein may be because they are hydrolysed into smaller peptides, which retain antifungal activity. The nature of active molecule may be peptide, polyketide, lipopeptide, phospholipid and others. [27,28]

Non-toxic nature is an important parameter in evaluation of a probiotic culture. Toxicity of the bacterial isolates SHW and COBT were tested by hemolytic assay using sheep blood agar and both were found to non-hemolytic in nature.

In order to check the ability of the culture to survive in intestinal conditions, the culture was subjected to bile salt hydrolysis and tolerance test using ox bile. The result showed that both cultures could tolerate 0.1 % to 2.0 %.bile salt for 6 hrs but failed to hydrolyze bile salt. Result show in Table No.2

Table 2. Bile tolerance test of Bacillus tequilensis cultures

Culture	TVC (x 104)						
	Control (without Ox bile)	OX bile concentration (%)					
		0.1	0.3	0.5	1	1.5	2
COBT	716	634	510	325	142	64	30
SHW	917	794	619	492	389	263	179

The antimicrobial properties of probiotic bacteria have been related to their metabolic products such as organic acids especially lactic acid, bacteriocins and hydrogen peroxide. There was appreciable production of the acid by the isolates; SHW produced 1.5042 g/L while COBT could produce 1.3512 g/L lactic acid in medium. Similarly these cultures also showed the ability to produce hydrogen peroxide [H_2O_2].

Nonoxinol-9 is safe contraceptive agent used to avoid pregnancy by killing sperms. A probiotic bacteria used as a vaginal suppository should also be able to tolerate spermicidal agents. [29] When exposed to Nonoxinol-9 at 5% concentration TVC of the cultures decreased to half with in 6 hrs while higher concentrations of nonoxinol-9 inhibited growth of the culture.

The isolates COBT and SHW were further subjected to 16sRNA sequencing and identified as *Bacillus tequilensis* with maximum similarity (99.86%) with KCTC 13622 (Accession no AYTO01000043).

DISCUSSION

Attempted study was to isolate bacteria that have anti-candida activity and are probiotics. Milk was selected as the source since many cultures isolated from milk has been reported as probiotics. In our study the cultures isolated from milk was screened for anti-candida activity and then for probiotic potential. In assays, supernatants of cultures showed best antimicrobial activity against *Candida albicans* ATCC 3557 was screened. Inhibition may be a part of protective mechanisms that allows probiotics to dominate in complex ecosystems, such as genital tracts or gastro-intestinal.

A probiotic bacterium should be able to survive acidic pH of stomach as well as alkaline pH of intestine. Result indicates that *Bacillus tequilensis* stains reported in the present could resist both conditions. Overall in the results, bile did not resist the growth of the bacteria completely and when subjected to 2% of bile, there were still a high number of bacteria. The high growth of the bacteria at 2% bile could be due to its stress adaptation mechanism. Both culture showed varying levels of survival in presence of different concentration of bile salt. This result indicates that bacterial isolates could most likely survive in the stomach and the small intestine, and colonize in the large intestine. The probiotic strains proved to exhibit an excellent quality of bile tolerance.

Another property evaluated in this work is the ability to tolerate high

temperature. Tolerance to high temperature would be an ideal character for an industrial culture. The reported cultures resisted high temperature as well as showed activity in the supernatant after treating at higher temperature. Although growth and activity of the bacteria decreased from 40^{o} to 80^{0} C they could survive for half hour even at 120^{0} C.

Anti-candida molecules reported from bacillus comprise predominantly of proteins. [28] So in order to find out the nature of the anti-candida molecule present in the isolate, the supernatant was subjected to proteinase K. The result showed that the active molecule is protein in nature since it lost complete activity after the treatment.

Toxicity is an important parameter in probiotic characterization. [1] In order to be assessed as probiotic a culture should be non-toxic and here the isolates did not show any hemolysis on sheep blood agar and hence are non-toxic in nature.

Organic acids, especially lactic acid are an important molecule since they can also inhibit pathogens. [28] The isolates were found to produce lactic acid at late stage of the growth. Similarly our study suggests that *Bacillus tequilensis* COBT and SHW have potential to produce H_2O_2 which play important role in controlling the pathogens.

Nonoxinol-9 is safe contraceptive agent used to avoid pregnancy by killing sperms. This compound at low concentrations (4%–16%) also kills useful bacteria present in vaginal micro flora. Any probiotic bacteria used as a vaginal suppository should also be able to tolerate spermicidal agents. [29] A probiotic culture should be able to survive and resist in vagina after exposure with vaginal suppository. Nonoxinol-9 demonstrates adverse effects on the normal vaginal flora, especially *Lactobacillus acidophilus.* [30] In our study *Bacillus tequilensis* treated with spermicidal agent Nonoxinol-9 at 5 % showed high tolerance. Hence, the culture would an ideal candidate for treating vaginal candidiasis in the form of vaginal suppository.

There are reports of anti-candida activity of *Bacillus tequilensis* however, its probiotic nature was never reported before. The present study indicates that *Bacillus tequilensis* SHW and COBT isolated from sheep milk and buffalo colostrums are ideal candidate for further probiotic characterization and will be useful in controlling diseases such as candidiasis. There is a trend in research towards natural remedies as alternative therapy to maintain human health, however, it needs more understanding how natural remedies work. This study provides additional facts to the amount of that knowledge in existence and hopes to encourage the acceptance, pursuit and development of a novel probiotic as therapeutic agents.

CONCLUSION

Nowadays use of probiotics has received a great attention as an alternative, inexpensive and natural remedy to restore and maintain health. Promising antifungal activity of *Bacillus tequilensis* isolated from sheep milk and buffalo colostrums can be utilized to restrain development of the pathogenic *Candida* spp. such as C. *albicans*. Major advantages of these non-hemolytic cultures are tolerance to heat, acidic pH, and bile salts which widens its potential as a probiotic culture. However, more investigations are needed to complete the identification of anti-candida bioactive compound from *Bacillus tequilensis* as well as to establish its probiotic nature.

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