

## Original Research Article

### Inhibition of pathogenic strains of *Candida albicans* and non-*albicans* by *Bacillus* species isolated from traditional Indian fermented food preparations

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#### A B S T R A C T

Total 25 bacterial cultures isolated from various food preparations using De Man Rogosa Sharpe (MRS) agar medium of pH 6.5 were screened for anti-candida activity by agar well diffusion assay against pathogenic isolates of 23 *Candida albicans* and 4 non-*albicans* strains, isolated from high vaginal swabs of pregnant ladies with vulvovaginal candidiasis. Five cultures isolated from *Meduwada* batter inhibited all the pathogenic *Candida albicans* and non-*albicans* used in the study. Optimum pH for maximum activity of anti-candida isolates varied from 5 to 10 after 76 h of incubation. These cultures showed specificity against *Candida* since they failed to inhibit other Gram positive and negative bacteria tested. Culture supernatant of the isolates could also inhibited the germ tube formation in *Candida albicans*, even after heat treatment at 60°C indicating the thermo-stability of the anti-candida molecule. The cultures were then subjected to 16sRNA gene sequencing and two of them were identified as *Bacillus tequilensis* with a homology of 99.4 and 99.5% and remaining three as *Bacillus subtilis* subsp. *spizizenii* with a homology of 99.68, 100 and 99.67. This study is the first report of inhibition of pathogenic *Candida albicans* and non-*albicans* and germ tube formation in *Candida albicans* by *Bacillus tequilensis*.

#### Keywords

Anti-candida,  
*Bacillus tequilensis*,  
*Bacillus subtilis*,  
fermented food

## Introduction

Vulvo vaginal candidiasis (VVC) is a common vaginal infection, majorly due to *Candida albicans*, characterized by itching, watery secretions and erythema and it has

been estimated that almost 75% of women get affected by VVC once in a life time (Marrazzo, 2003). Due to elevated estrogen and glycogen content in vaginal secretions,

risk of VVC increases during pregnancy and it can induce systemic infection in neonate (Cotch *et al.*, 1998; Filippi *et al.*, 2009). Studies have proven that treatment for VVC during pregnancy with clotrimazole or fluconazole have considerably reduced preterm birth in many candidates (Roberts *et al.*, 2011; Soong & Einarson, 2009). However, reports on the side effects of these drugs as well as increase in drug resistance in *Candida albicans* highlight the importance of finding new drugs against the pathogen (Lewis, 2011; Pfaller, 2012; Rathod *et al.*, 2012). Moreover, there are reports of candidiasis due to non-albicans strains such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. dubliniensis* and *C. guilliermondii* as well as their resistance to commonly used drugs (Jordán *et al.*, 2014; Pfaller *et al.*, 2014; Silva *et al.*, 2012). Bacteria have always been an important source of anti-microbial compounds and many Gram positive bacteria are reported for the ability to inhibit *Candida albicans* (Cruz *et al.*, 2013; Hassi *et al.*, 2012; Jarosz *et al.*, 2009). The Present study reports the isolation and identification of anti-candida *Bacillus* species from fermented food preparations, screening their activity against pathogenic *Candida albicans* and non-albicans such as *Candida rugosa*, *Candida glabrata* and *Candida parapsilosis*, as well as inhibition of germ tube formation in *Candida albicans* by the bacterial isolates.

## Materials and methods

### Samples and isolation

Fermented batter of Indian traditional food items such as *Idli*, *Meduwada* and *Jalebi* as well as dairy products such as curd from sheep and cow milk were used for isolation of bacteria. Samples (1 g or 10 ml ) suspended in 100 ml saline were serially

diluted and plated in MRS agar (Himedia, Mumbai, India) medium of pH 6.5 (Agaliya & Jeevaratnam, 2013). The plates were incubated at 37°C for 24 h, after the incubation, morphologically dissimilar bacterial colonies formed on the plates were selected and then purified by streaking on MRS agar plates. The pure colonies were sub-cultured in MRS agar slants and stored in refrigerator for further study. All experiments were repeated three times and average was taken as the final reading.

### Screening of anti-candida activity

Anti-candida activity of the isolates was screened against pathogenic *Candida albicans* strain CA10 obtained from Bharati Vidyapeeth Deemed University Medical College culture collection, Pune, India. Using agar well diffusion anti-candida activity of the isolates was screened against 27 pathogenic *Candida albicans* and non *albicans* strain isolated from high vaginal swabs of pregnant ladies with vulvovaginal candidiasis at Bharati Vidyapeeth Deemed University Medical College, Pune, India. *Candida* culture (0.5 OD A<sub>600</sub>, 100 µl) were plated on Sabouraud's dextrose agar plates and incubated for 30 min (Letscher-Bru *et al.*, 2013). Bacterial suspensions (10 µl, 1 OD) were added in wells (3 mm dia.) made on the agar followed by incubation at 37°C for 24 h. 20µl fluconazole (1.25 mg/ml) was added in each well as the positive control (Magaldi *et al.* 2004).

Anti-candida activity was scored by measuring the diameter of zone of inhibition around the wells. Cultures showing positive activity were then screened against pathogenic isolates of *Candida albicans* (23 nos.) and non-albicans strains (4 nos.), isolated from high vaginal swabs of pregnant ladies with VVC at the OPD of Bharati Hospital, Pune, India, using

CHROM agar and identified by VITEK<sup>®</sup> 2 compac as well as by performing germ tube test (Chan *et al.*, 2011; Melhem *et al.*, 2013; Nejad *et al.*, 2013).

### **Optimization of incubation time and pH**

To find out the optimum pH for maximum growth and inhibition, the Sabouraud's dextrose broth of pH range 2-10 was inoculated with 1.0 OD, 1% bacterial cultures and incubated at 37°C on rotary shaker for 76 h before checking the growth and anti-candida activity. Similarly maximum activity and growth were checked at regular intervals by incubating the culture at optimum pH for 92 h (Augustine *et al.*, 2004).

### **Germ tube inhibition studies**

Germ tube inhibition studies were conducted according to Brayman and Wilks, 2003. The method was modified as follows: *Candida* cultures (100 µl) in potato dextrose broth containing 0.2% human serum were added in treated 96-well flat-bottom plates followed by the addition of 100 µl, 1.0 OD saline suspension of bacterial isolates or culture supernatant with and without heat treatment at 60°C for 1 h. The plate was incubated at 37°C for 4 h to induce germ tube formation. *Candida* with saline was used as negative control. After the incubation the medium in the wells were discarded by inverting the plate and each well was washed with 70% ethanol followed by 25% SDS and three times with distilled water followed by staining with 200 µl of 0.1% crystal violet (10 min.). The plate was then washed thrice with distilled water three times followed by sterile 0.25% SDS and thrice with sterile distilled water. Plates were dried and a mixture of 200 µl of isopropanol with 0.04 N HCl and 50 µl 0.25% SDS were added to elute the crystal

violet. Absorbance of the eluted dye was determined at 590 nm using an ELISA plate reader (Biotek, India). The germ tube inhibition was measured in percentage with respect to negative control.

### **Bacterial identification by 16S rRNA gene sequencing**

Total genomic DNA was isolated using Gene Elute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl<sub>2</sub>; 1 x PCR buffer; 200 µM each dCTP, dGTP, dATP and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (Sigma, USA) in a final volume of 20 µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene, as described previously (Rawlings, 1995). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94°C for 5 min; 30 cycles of 94°, 60°, and 72°C for 1 min each; final extension at 72°C for 10 min. The mixture was held at 4°C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice-using 70% ethanol and dissolved in Tris-HCL (10mM, pH 8.0).

The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, California, USA) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the 16S rRNA gene (Muyzer *et al.*, 1993; Rawlings, 1995). The sequencing reaction and template preparation were performed and purified in accordance with the directions of the manufacturer (Applied Biosystems, USA). Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems,

USA). The sequencing output was analyzed using the accompanying DNA Sequence Analyzer software (Applied Biosystems). The sequence was compared with reference sequences available in GenBank using the BLAST algorithm.

## Result and Discussion

### Isolation of bacteria and Screening of anti-candida activity

Out of 23, Five Gram positive cultures designated as Paci, MW3, MW9, MW27 and MW31 were isolated from *Maiduwada* dough, inhibited pathogenic *Candida* (Figure 1). *Maiduwada* dough used in the study is fermented dough made from mixture of rice flour and black gram flour, traditionally used for the preparation of a snack called as *Maiduwada* or *Uditwada* (Sarkar, 2014). Many Gram positive bacteria have been isolated previously from traditional fermented products and their anti-candida properties were documented (Jeygowri *et al.*, 2014; Ndagano *et al.*, 2011; Tropcheva *et al.*, 2014). This is the first report of isolation of anti-candida bacteria from *Maiduwada* dough.

### Identification of isolated bacteria

Cultures were subjected to 16 sRNA gene sequencing and MW3 and MW9 showed closest phylogenetic affiliation towards *Bacillus tequilensis* with a homology of 99.4 and 99.57% respectively. While MW27, MW31 and Paci showed closest phylogenetic affiliation towards *Bacillus subtilis* subsp. *spizizenii* with a homology of 99.68, 100 and 99.67% respectively.

### Optimization of incubation time and pH

Optimum pH for growth and *Candida* inhibition for the isolates varied from pH 6

to 10. Culture MW27, 31 and Paci showed growth at pH 5 to 10 with maximum growth and inhibition at pH 10. The range of pH for MW3 was 3-10 with optimum pH at 6 and for MW9 the range of pH was 4 –10 with optimum pH at 7. Time optimization studies showed anti-candida activity increased along with growth and reached maximum at 76 h for all cultures and remain same till 92 h. Ability to survive in highly acidic pH is one of the important characters required for probiotic bacteria (Tuomola *et al.*, 2001) and MW3 and 9 showed this property, hence are ideal candidates for probiotic characterization studies.

Under optimal conditions bacterial isolates could inhibit all *C. albicans* strains tested in the study. The efficiency of the inhibition was highest for MW31 followed by Paci, MW27, MW3 and MW9 (Table 1). Considering the spread of these pathogens, an anti-fungal drug should have the ability to inhibit various species of pathogenic *Candida*. It is clear from the table 1 that all bacterial isolates could inhibit non-*albicans* strains such as *C. parapsilosis*, *C. rugosa* and *C. glabrata*.

Although, VVC is an infection majorly caused by *C. albicans*, infection due to non-*albicans* are also reported by many researchers (Jordán *et al.*, 2014; Pfaller *et al.*, 2014). *B. tequilensis* and *B. subtilis* subsp. *Spizizenii* could inhibit both *C. albicans* and non-*albicans* pathogens such as *C. parapsilosis*, *C. rugosa* and *C. glabrata* indicating that these isoaltes could be useful in development of drugs against a range of *Candida* species. *B. subtilis* is known for the production of many anti-microbial and anti-fungal proteins as well as lipo-peptides (Ramachandran *et al.*, 2014; Tan *et al.*, 2013). However, *B. tequilensis* was never reported before for its anti-candida property.

**Germ tube inhibition studies**

Germ tube formation is a very important stage in the formation of biofilm in *C. albicans* (Ramage *et al.*, 2005). Germ tube have been shown to contribute virulence in models of skin and mucosal infections (Mayer *et al.*, 2013) and an ideal anti-candida drug should be able to inhibit the formation of germ tube in *C. albicans*. Germ tube inhibition assays showed that both

bacterial suspension and supernatant were capable of 100% inhibition of germ tube formation (Table 2). The culture free supernatant could inhibit germ tube formation even after heating at 60°C for 1 h, indicating the thermostability of the anti-candida molecule (Lin *et al.*, 2009; Skouri-Gargouri *et al.*, 2008). Previous reports showed that many heat stable peptides isolated from *B. subtilis* showed antimicrobial activity.

**Table.1** Activity of isolated bacteria against multiple *Candida* isolates

Candida strains	Bacterial isolates				
	MW27	MW3	MW31	MW9	Paci
CA-1	1.9	2.8	3	1.7	3.1
CA-2	2.3	2	3	1.9	2.6
CA-4	0.7	-	1.2	-	0.9
CA-5	1.5	1.6	2.1	1.5	2
CA-9	1.6	1.6	2.2	1.1	2.1
CA-10	1.2	1.1	2.1	0.6	2.2
CA-13	1.2	1.2	2.2	1.1	2.1
CA-14	2	2.1	2.7	2.1	2.4
CA-15	2.8	1.2	2.4	1.5	2.1
CA-16	2	1.3	3.2	1.5	2.1
CA-18	2.8	1.9	2.0	2.3	1.6
CA-20	2.6	1.2	2.8	1.2	2.1
CA-22	2.3	1.4	1.5	1.0	1.2
CA-23	2.8	-	2.3	1.0	2.2
CA-26	1.9	1.5	3	1.5	2.9
CA-27	2.7	1.7	2.2	1.5	2.2
CA-28	1.7	1.2	2.2	1.3	2.1
CA-32	2.7	1.7	2.2	1.4	2.2
CA-33	2.7	1.4	2.8	1.9	2.4
CA-34	2.3	1.6	2.0	1.3	1.7
CA-35	2.4	1.8	3	2.4	2.5
CA-36	1.2	2.2	2.7	1.1	2.1
CA-38	1.8	2.5	2	1.3	2.7
CG-11	-	-	-	0.7	-
CG-21	0.7	-	-	-	-
CP-37	-	-	1	-	1
CR-ACNA	-	-	0.9	-	1

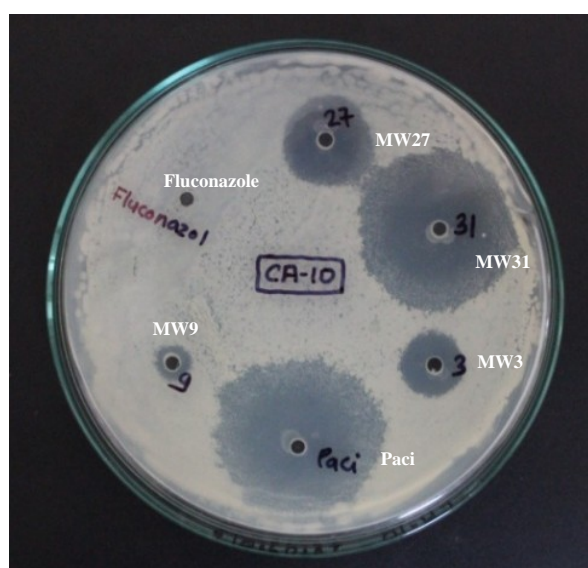
CA- *Candida albicans*, CR- *Candida rugosa*, CG- *Candida glabrata*, CP- *Candida parapsilosis*.

**Table.2** Germ tube inhibition (%) by isolates

	MW27	MW31	MW3	Paci	MW9
CA	0	0	0	0	0
CA+ Bacterial suspension	95	99	100	97	98
CA+ Supernatant	99	99	100	100	100
CA+ heat treated Supernatant	93	99	95	96	94

CA- *Candida albicans*

**Figure.1** Inhibition of *Candida albicans* CA10 by isolates MW3, MW9, MW27, MW31 and Paci



Due to the range of anti-candida activity, ability to inhibit germ tube formation, thermostability and resistance to acidic pH makes *B. tequilensis* and *B. subtilis* subsp. *Spizizenii* isolated from *Maiduwada* dough, an ideal candidate for drug discovery study. However, further studies are needed to purify and understand the anti-candida molecule with respect to its nature, toxicity and other pharmacological characters.

In the present study bacterial strains were isolated from various traditional fermented products and screened against pathogenic *C.*

*albicans* and non-*albicans* isolated from pregnant women with VVC. From *Maiduwada* dough used in the study five gram + anti-candida bacteria were isolated and they were identified as *Bacillus tequilensis* 1, 2 and *Bacillus subtilis* subsp. *Spizizenii* 1,2 and 3. This is the first report of anticandida activity of *Bacillus tequilensis*. All five anti-candida cultures showed maximum activity when grown at 37°C for 76 h. All cultures could grow at acidic as well as basic pH and hence are ideal for probiotic characterization studies. Probiotics have been reported and

highlighted as alternative therapy for VVC during pregnancy. Culture supernatant could inhibit formation of germ tube in *Candida albicans* even after heat treatment which implies the thermostability of the anticandida molecule secreted by the bacterial cells.

Currently used anti-fungal drugs are known to inhibit germ tube formation, however, the side effects of these drugs limit their use during pregnancy (Lamont *et al.*, 2014; Pilmis *et al.*, 2014). Moreover, due to drug resistance in *Candida* there is large scale search for new biological compounds that are safe and effective in treating candidiasis (Pfaller, 2012; Rathod *et al.*, 2012). *B. tequilensis* 1 and 2 as well as *B. subtilis* subsp. *Spizizenii* 1,2 and 3 isolated from *Maiduwada* are ideal candidate for finding new drugs against pathogenic *Candida* due to the thermostable nature, germ tube inhibition and range of activity against various *Candida* species. However, further studies are needed to purify and understand the anti-candida molecule with respect to its nature, toxicity and other pharmacological characters.

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