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RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF *BREVIBACTERIUM*

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Abstract

Screening the microorganisms for development of the antimicrobial agent against the infectious diseases was developed in clinical field. In the present study the antimicrobial activity of *Brevibacterium* was tested against *E. coli*, *Bacillus polymyxa, Alternaria*, *Candida albicans*, *S. cerevisiae*, *S. boulardii* and *Malassezia furfur*. Antimicrobial activity of *Brevibacterium* was observed against *S. cerevisiae* and *Candida albicans.* Kill curve with *Brevibacterium* and supernatant of *Brevibacterium* against cultures *S. cerevisiae* and *Candida albicans* were performed. Kill curve method will help to know the mode of action of *Brevibacterium* and supernant of *Brevibacterium* against *S. cerevisiae* and *Candida albicans*. Antimicrobial activity of supernatant against *S. cerevisiae* was observed at 1.5C from 0minute. But antimicrobial activity of supernatant against *Candida albicans* was observed at 1.5C after 90minutes*.* EtBr efflux assay test was performed against *Candida albicans* and *S. cerevisiae*. From the result we concluded that *Brevibacterium* affect the cell wall of *S. cerevisiae* and enzyme of *Candida albicans*.

Keywords: *Brevibacterium,* Kill curve, antimicrobial activity, EtBr efflux assay test

Introduction

Treatment of infectious diseases caused by pathogenic bacterial and fungal strains was one of the most traditional problems in the clinical field (Mehrgan et al., 2008; Fazly Bazzaz et al., 2005; Calvin, 1993). This necessity encouraged the investigators to synthesize novel and more potent inhibitory compounds (like azoles and quinolones derivatives) (Emami et al., 2008; Shafiee et al., 2008) to fight them. However, the adverse effects and also appearance of bacterial or fungal resistances persuaded the investigators to study on natural products from microorganisms or herbal extracts to discover novel and safe lead compounds (Mehrgan et al., 2008; Fazly Bazzaz et al., 2005). To reach this approach, screening of fungal and bacterial strains able to produce inhibitory compounds is the first step in the discovery of novel antibiotic compounds (Imada et al., 2007). Marine microorganisms and also cyanobacteria were found to produce such inhibitory compounds (Imada et al., 2007; El-Sheekh et al., 2006), but most of the available antibiotics are originally produced by terrestrial fungal and bacterial strains (Mannanov and Sattarova, 2001).

Brevibacterium is a genus of actinobacteria of the order Actinomycetales*.* It is the sole genus in the family Brevibacteriaceae. The genus is a heterogeneous mixture of coryneform organisms that have particular application to industrial production of vitamins, amino acids for fine chemical production, and are commonly used in cheese production (Seefeldt and Weimer, 2000). *Brevibacterium* was also a lactic acid bacterium. *Brevibacterium* can produce Bacteriocins and thiol. Bacteriocins are bacterial peptides that are

inhibitory to micro-organisms that are usually, but not always, closely related to the producer strain (Klaenhammer 1988). Because of their potential use as natural preservatives, bacteriocins produced by lactic acid bacteria have been the subject of intensive investigation in recent years (Klaenhammer 1988; Nettles and Barefoot 1993). *Brevibacterium* shows activity against a wide variety of moulds and yeasts (Lewis 1985). *Brevibacterium* species produce thiol and these thiols convert methionine into Methanethiol. Methanethiol from *Brevibacterium* possess antifungal activity.

Antimicrobial activity of *Brevibacterium* has been tested against *E. coli* (Gram positive bacteria), *Bacillus polymyxa* (Gram negative bacteria), *Alternaria* (Mould), *S. cerevisiae, S. boulardii, Candida albicans* and *Malassezia furfur* (Yeast). The mode of action of *Brevibacterium* and supernatant of *Brevibacterium* were tested.

Materials and Methods

Organisms

The bacterial isolate which was isolated from food sample at T Stanes and Company Limited, Coimbatore, Tamilnadu was maintained in Tryptone soya medium.

Pathogens used

The bacteria and fungi culture used in the present study include *E. coli*(Gram positive bacteria), *Bacillus polymyxa* (Gram negative bacteria), *Alternaria*(Mould), *Candida albicans, S. cerevisiae*, *S. boulardii* and *Malassezia furfur* (Yeast) isolates were obtained from T Stanes and Company Limited, Coimbatore, Tamilnadu. The bacterial cultures were maintained in Nutrient broth media. Mould culture was maintained in potato Dextrose Agar. *Candida albicans*, *S. cerevisiae*, and *S. boulardii* were maintained in Sarbouraud Dextrose Broth. *Malassezia furfur* was maintained in Dixon's Agar.

Screening of antimicrobial assay

Antimicrobial activity of *Brevibacterium* was tested against *E.coli* (Gram positive bacteria) and Bacillus polymyxa (Gram negative bacteria), *Alternaria* sp. (Mould) and *Candida albicans*, S .*cerevisiae*, *S. boulardii* and *Malassezia furfur* (Yeast). Pour 20ml of Mueller Hinton Agar in two petriplates and allow it solidify for 30 minutes. Overlay 5ml of MHA which contain 50micro liter of test culture (*E. coli* and *Bacillus polymyxa*) into to two 20ml molten MHA plates and allow it to solidify for 30 minutes. Inoculate loopful of *Brevibacterium* culture in the center of MHA plates and then incubate the plates at 37 degree Celsius for 24 hours. Pour 20ml of Sarbouraud Dextrose Agar in four petriplates and allow it solidify for 30 minutes. Overlay 5ml of SDA which contain 50micro liter of test culture (*Candida albicans*, *S. cerevisiae*, and *S. boulardii*) into to four 20ml molten SDA plates and allow it too solidly for 30 minutes. Inoculate loopful of *Brevibacterium* culture in the center of SDA plates and then incubate the plates at 30 degree Celsius for 24 hours.

Pour 20ml of Dixon's Agar in four petriplates and allow it solidify for 30 minutes. Overlay 5ml of Dixon's Agar which contain 50micro liter of test culture (*Malassezia furfur*) into to four 20ml molten Dixon's Agar plates and allow it to solidy for 30 minutes. Inoculate loopful of *Brevibacterium* culture in the center of Dixon's Agar plates and then incubate the plates at 30 degree Celsius for 24 hours.

Pour 20ml of Potato Dextrose Agar in four petriplates and allow it solidify for 30 minutes. Take loopful of test culture Alterneria and spread it on the Agar and allow it fix for 5 minutes. . Inoculate loopful of *Brevibacterium* culture in the center of Dixon's Agar plates and then incubate the plates at 30 degree Celsius for 24 hours.

Kill curve with *Brevibacterium*

Kill curve assay was performed in heamocytometer. Add 100microliter of *Brevibacterium* and yeast (*Candida albicans* and *S. cerevisiae*) in 20ml Nutrient broth with Dextrose. At 0 hour take 10microliter of culture and load the culture in hemocytometer and for makes uniform distribution, place cover slip over it. View the cells under a microscope at 40x magnification. Cells were counted in 4 outer square (diameter 1ml) and centre in the grid. And then incubate the broth for 4 hours and then count the cells in heamocytometer. Likewise cells were counted at 6th hour and 8th hour. Likewise draw the kill curve for the control (100ml *Brevibacterium* in 20ml Nutrient Broth and 100ml yeast (*Candida albicans* and *S. cerevisiae*) in SDB) in the hemocytometer.

Kill curve with supernatant

Collect the supernatant by centrifuge the media contain both *Brevibacterium* and yeast (*Candida albicans* and *S. cerevisiae*) at 6000 rpm for 20 minutes. Add 0.5ml, 1.0ml &1.5ml supernatant and 50microliter yeast culture (*Candida albicans* and *S. cerevisiae*) 10ml SDB. At 0 minute, spread plate was done for three different concentrations in SBA in petriplate. Likewise spread plate done at 15minute, 30minute, 45minute, 60minute, 90minute, 120minute, 150minute, 180minute, 210minute and 240minute. Three controls were made for three different concentrations. Incubate the all the plates at 30 degree Celsius for 24 hours.

Alternatively, at 0 minute, cells were counted for 1.5ml supernatant with 50microliter yeast (*Candida albicans* and *S. cerevisiae*) in 10ml SDB. Likewise cells were counted at 15minute, 30minute, 45minute, 60minute, 90minute, 120minute, 150minute, 180minute, 210minute and 240minute. Cells also counted for the control, Yeast (*Candida albicans* and *S. cerevisiae*) in 10ml SDB.

EtBr efflux assay

Mid logarithmic phase yeast (*Candida albicans* and *S. cerevisiae*), grown in SDB medium were loaded with 10microgram of EtBr/ml in the presence of 200microliter of supernant of broth contain both *Brevibacterium* and Yeast (*Candida albicans* and *S. cerevisiae*). Cells were incubated at 37 degree Celsius for 20 minutes and then pelleted by centrifugation. The medium was decanted and the cell pellet was resuspended in the fresh SDB medium. To an optical density at 600nm of monitoring fluorescence at excitation and emission wavelengths of 530 and 600nm in a black 96 well polystyrene plate with a clear, flat bottom by using a spectromax germini spectroflurimeter.

Results

The Antimicrobial activity of *Brevibacterium* was tested against *E.coli, Bacillus polymyxa, Alternaria* sp., *Candida albicans*, *Malassezia furfur*, *S. cerevisiae*, and *S. boulardii*. Antimicrobial activities of *Brevibacterium* against test cultures were tested in agar plates. After 24hours of incubation, inhibition of *Brevibacterium* against test cultures was checked. *Brevibacterium* shows Antifungal activity against *Candida albicans* and *S.cerevisiae* (Zone of inhibition of observed was 3.4mm in the diameter) after the growth in 24hours. But *Brevibacterium* does not show any activity against *E.coli, Bacillus polymyxa,* Alternaria sp., *Malassezia furfur* and *S. boulardii*.

The Kill curve method was done for identifying the activity of *Brevibacterium* against *Candida albicans* and *S.cerevisiae* in different time interval of 0hour, 4 hour, 6 hours and 8 hours in heamocytometer. The antifungal activity of *Brevibacterium* against *Candida albicans* and *S. cerevisiae* was observed at 6hour and 8 hour. The graph for kill curve was given in figure 1 and 2:

The kill curve method was done for identifying the activity of supernatant of *Brevibacterium* and Yeast (*Candida albicans* and *S. cerevisiae*) in the same medium against *Candida albicans* and *S.cerevisiae*. Kill curve was done in different time interval of 0minute, 15minute, 30minute, 45minute, 60minute, 90minute, 120minute, 150minute, 180minute, 210minute and 240minute in plate count method and heamocytometer. For *Candida albicans*, high antifungal activity was observed in 1.5c at 90minute in both plate count and heamocytometer. For *S. cerevisiae*, high antifungal activity was observed for 1.5c from 0minute to 120minute in plate count and hemocytometer. The result of kill curve with supernatant against *Candida albicans* and *S. cerevisiae* were shown in figure 3 and 4.

EtBr efflux assay

The EtBr efflux assay was tested for *Brevibacterium* with yeast culture (*Candida albicans* and *S. cerevisiae*) at 0minute, 5minute, 10minute, 15minute and 20minute results were given in table 1 and 2.

Discussion

Antimicrobial activity was tested against *E.coli, Bacillus polymyxa,* Alternaria, *S. cerevisiae*, *S. boulardii*, *Candida albicans* and *Malassezia furfur*. The strong antimicrobial activity was observed against *Candida albicans* and *S. cerevisiae*. *Brevibacterium* shows activity against *Candida albicans* and *S. cerevisiae* at after the incubation of 8 hours. But supernatant of *Brevibacterium* and *S. cerevisiae* culture shows antifungal activity at 0minutes to 120 minutes against *S. cerevisiae*. But supernatant of *Brevibacterium* and *Candida albicans* culture shows antifungal activity at 90minutes and 120minutes against *Candida albicans.*

In this case, *Brevibacterium* produce some enzymes and peptides (Bacteriocins and thiol). Those compounds were responsible for the inhibit of the growth of *S. cerevisiae* and *C.albicans.*

Figure 1: Kill curve for *Candida albicans* with *Brevibacterium*

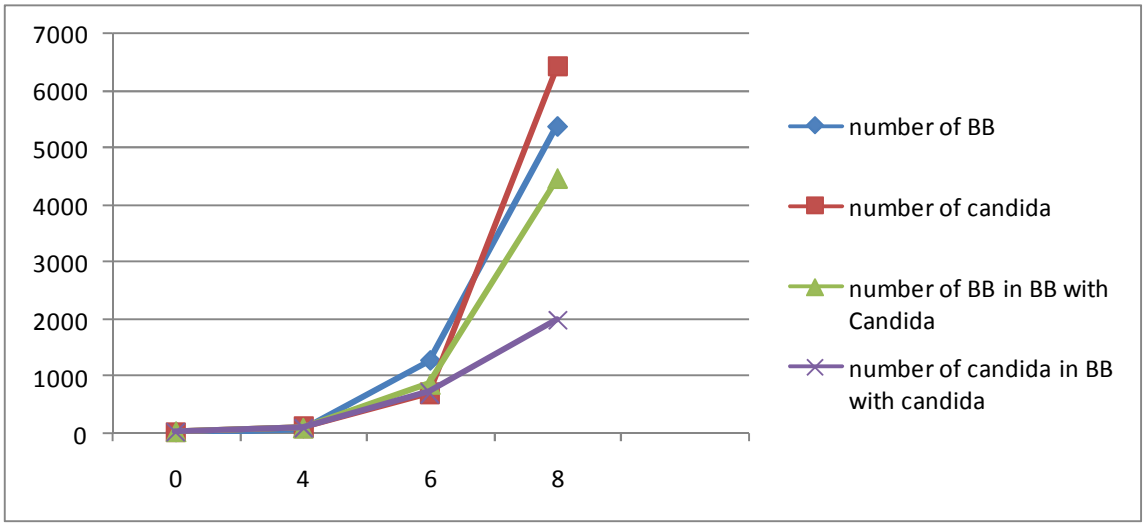

Figure 2: Kill curve for *S. cerevisiae* with *Brevibacterium*

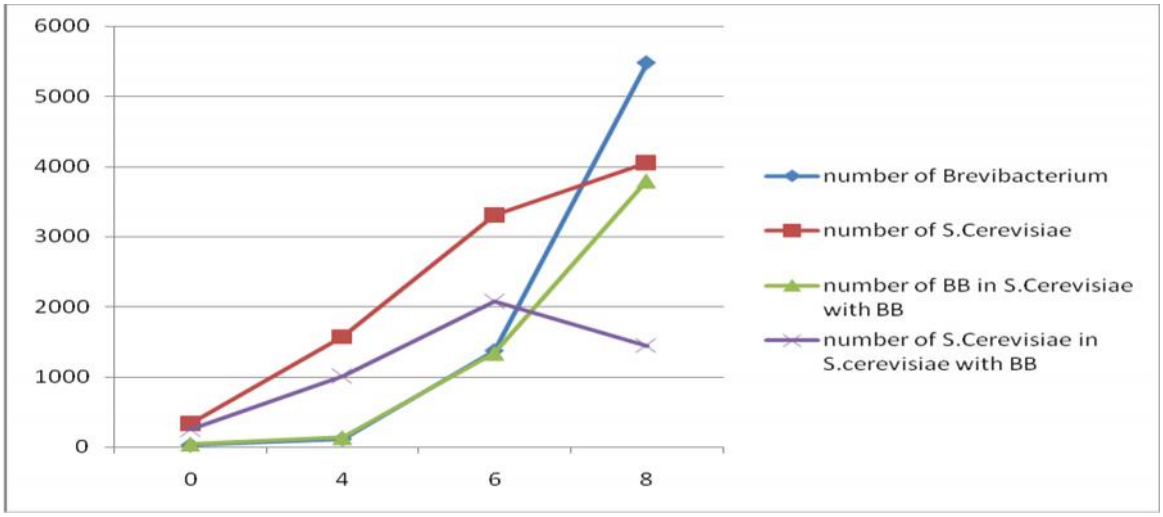

Figure 3: Kill curve for *Candida albicans* with supernatant

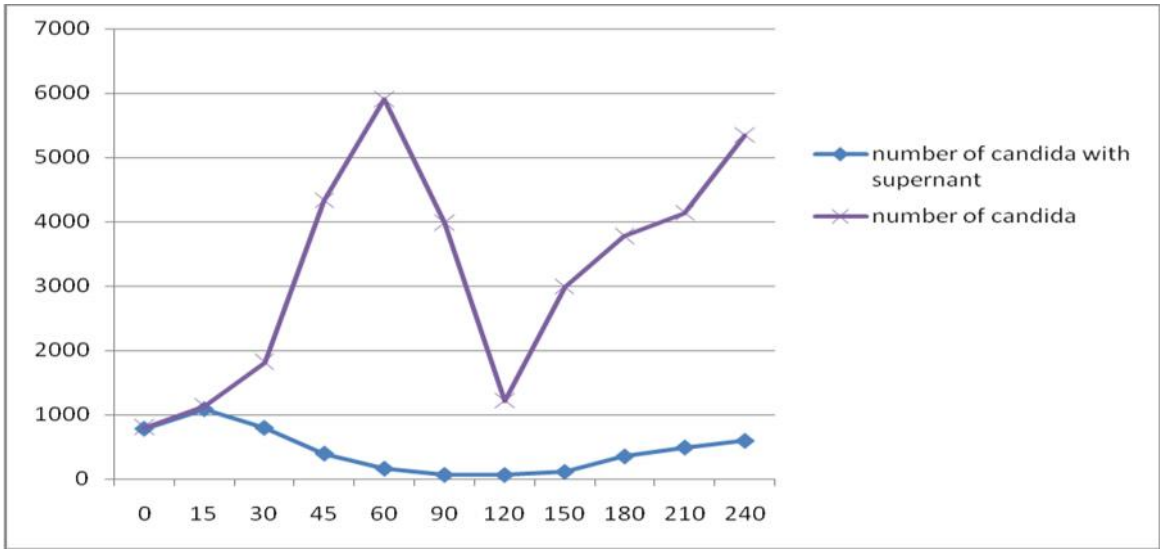

Figure 4: Kill curve for *S. cerevisiae* with supernatant

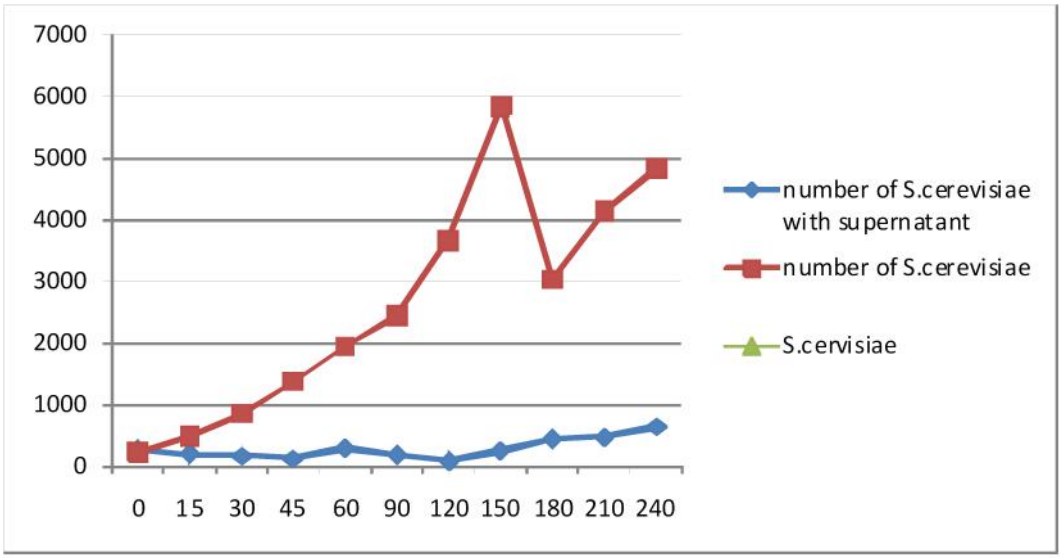

Table 1: EtBr efflux assay result for *Candida albicans*

***S*.No**	**Control (No EPI)**	**Positive (EPI)**	***Candida albicans***
	1	1	1
1	0.6413	0.8247	0.6017
2	0.5707	0.7842	0.5501
3	0.5343	0.7134	0.5216
4	0.5108	0.7011	0.4978
5	0.51	0.6892	0.4972

Table 2: EtBr efflux assay result for *S. cerevisiae*

S.No	**Control (No EPI)**	**Positive (EPI)**	***S. cerevisiae***
	1	1	1
1.	0.7112	0.8336	0.7106
2.	0.6941	0.7731	0.64
3.	0.5532	0.7023	0.5505
4.	0.5297	0.69	0.5067
5.	0.4026	0.6781	0.4961

Affect of supernatant of *Brevibacterium* on *S.cerevisiae* was observed at the 0 minute onward, because enzymes from the *Brevibacterium* affect the cell wall of *S.cerevisiae* and inhibit the growth of growth of *S.cerevisiae*. But affect of supernatant of *Brevibacterium* on *Candida albicans* takes 90minites*.* Because the enzymes from the *Brevibacterium* affect some enzymes in the *Candida abicans* and inhibit the growth of *Candida albicans.*

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