SCREENING FOR POTENT MICROBIAL ANTAGONISTS AS AN ALTERNATIVE FOR THE CONTROL OF CANDIDA

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ABSTRACT

Candida albicans is a potent opportunistic human pathogen causing systemic as well superficial infection in mucous membranes of gastrointestinal and urinary tracts. It is the most common cause of vulvovaginitis in females as well as oral thrush. The search for novel strain of Bacillus subtilis from complex habitat having anticandidal activity would be an effective alternative to combat against the drug resistance. The isolate UK-3, later identified as Bacillus subtilis, showed antimicrobial activity during the screening process. Extract from the fermented broth by ethyl acetate showed anticandidal activity by agar diffusion method.

Keywords: ***Anticandida activity, Antifungal compound, Bacillus subtilis, Candidiasis, Vulvovaginitis.***

I. INTRODUCTION

Candida usually occurs as a normal flora of the human body but it can occur as an opportunistic pathogen causing superficial as well as systemic infection[1]. The pathogenesis of candidial infections is responsible for different types of disease manifestations. These pathogenic yeasts invade the mucous membrane and causes candidiasis in immunocompromised persons and the people who are at a greater risk are those suffering from HIV, cancer and those undergoin organ transplantation[2,3]. Among the 163 species of candida occurring on different habitat, Candida *albicans* is the common cause of oral thrush, candidiasis, candiduria as well as vulvovaginitis in females[2,4]. The prevalence of *C. albicans* is accounted to 70-90% while the other species is rarely isolated from clinical samples. In one study out of the total yeast isolated 47.6 % werepredominantly C. *albicans*.The percent occurrence of *Candida albicans* in India accounts up to 43% in the last ten years [4].

The Bacillus group of organisms as a potent producers of polypeptide antibiotics. Out of the 29 species of Bacillus isolated from soil most of them showed inhibition against gram positive and gram negative bacteria[5]. These organisms produces various classes of bacteriocins[6], surface-active biosurfactants[7] which includes lipopeptides and glycopeptides like inturins[8], surfactins[9], fengycins[10], kurstakins[11], bacillomycins[12] and mycosubtilin[13]. The antibiotics producing species mainly belong to *B. subtilis, B. polymyxa, B. brevis, B. licheniformis, B. circulans and B. cereus.* These group of organisms have been extensively exploited to produce antibiotics which are effective mostly for the gram positive bacteria, However, the production of large spectrum antibiotics as well as anti-fungal antibiotics producing organisms are relatively very less[14]. Moreover the available antifungal drugs are either ineffective against some fungi or may develop drug resistance resulting into no cure for variety of fungal infections. Candida species are important human pathogen and these organisms differ in their susceptibility against the common available antifungal agents[15]. Out of the total 213 isolates from the clinical samples the most common are *Candida tropicalis* (56%) followed by *Candida albicans*

(33%)[16]. A huge potential is been observed in the Bacillus strains due to their antimicrobial activity against pathogens[17] due to its activities of low toxicity and biodegradability. The search of novel microorganism as well as its products specifically Bacillus from microbiologically unexplored ecosystems is still an area of interest.

II. MATERIAL AND METHODS

2.1 Sample Collection

Soil samples were collected from the agricultural fields surrounding Navsaricity, Gujarat, India. Soil samples were collected from the rhizospheric soil area in sterile polyethylene bag, tightlypacked and immediately transferred to Microbiology Laboratory of NLCPAS, Navsari and stored in refrigerator till use.

2.2 Isolation of Microorganism

2.2.1 Isolation of Microorganisms from Soil Samples.

All these soil samples were serially diluted and spread on Nutrient agar plates containing 0.5% peptone; 0.3% beef extract; 1.5 % agar; 0.5% NaCl; and the pH was adjusted to 7.2 soil samples for isolation and enumeration. Plates were incubated at 37°C for 24 hr. After incubation the isolated bacterial colonies were studied and subjected for the screening of anticandidal activity.

2.2.2 Isolation of Candida Albicans from Clinical Samples

Urine samples from the patients suffering from urinary tract infection were collected from Advance Diagnostic laboratory, Surat and organisms were isolated and identified by growing them on CHROMagar media and further identified by BD Pheonix^{TM}.

2.3 Screening of Antagonistic Strains by in Vitro Antagonism Experiments

Screening of antagonistic activity of the soil isolate was carried out by spreading (except the center) the *Candida albicans* obtained from the clinical samples on the Nutrient Agar plates. Each isolated microorganism was streaked in circular form in the center of plate. Inoculated plates were incubated at 37°C for 24- 48 hr. Screening was done on the basis of the zone of inhibition produced by the soil isolate.

2.4 Identification of Isolate

The identification of the soil isolate, giving zone of inhibition against *Candida albicans*, was carried out from morphological, cultural and biochemical study. Further identification of the isolate was done on the BD Pheonix^{TM}.

2.5 Production and Extraction of Antifungal Compound

A loopful of purified culture of the isolate was inoculated aseptically in 100 ml of nutrient broth (pH 7.2) and incubated at 28°C in shaker incubator at 150 rpm for 72 hr. After incubation, the fermented broth was centrifuged to remove the bacterial cells and further filtered. The filtrate was mixed with ethyl acetate in the ratio of 1:1 (v/v) and shaken vigorously for 1 hr in a solvent extraction funnel. The solvent phase was separated from the aqueous phase and evaporated to dryness in water bath at 80° - 90°C and the residue was used to assess the anticandidal activity.

2.6 Study of Anticandidal Activity

Determination of antifungal activities of pure bacterial culture was performed by using agar well diffusion method. Wells were made in the agar medium and filled with 200 µl of extract. To determine the effect of the extract, *Candida albicans* was spread on the surface of sabouraud dextrose agar and incubated at 28°C for 48-72hr. Control plates were prepared in the same way without addition of the extract. After the incubation diameter of zone of inhibition was measured.

III RESULTS & DISCUSSION

3.1 Isolation & Identification of Soil Isolate

Bacillus group of organisms isolated from complex habitat are most abundant producers of antimicrobial peptides. Multiple strains are found to produce antimicrobial peptides which exhibit selective inhibition. Many of these strains are reported to produce wide variety of biosurfactants like inturins or surfactins[18]. Out of the 25 isolates obtained from the soil sample, 5 isolates showed antimicrobial activity during the screening process. Out of these five isolates, Isolate UK3 was observed to be most potent in having antimicrobial activity having maximum zone of inhibition. Hence the UK3 isolate (Fig.-1) was further identified to be *Bacillus subtilis* from the morphological characteristics showing gram positive rods, the colonies were large, flat and become creamy after 48 hours. The further identification by Pheonix™ confirmed the isolate UK3 to be *Bacillus subtilis.*

Fig.-1: Cultural Characterization of The Soil Isolate Showing Antagonistic Activity.

3.2 Isolation of Candida Albicans from Clinical Samples

Fifty urine samples were analyzed microbiologically obtained from patients suspected from suffering from UTIs. The suspected organisms were isolated and identified as per the morphological, cultural, biochemical characteristics. Twenty urine samples showed the presence of pathogens out of which the presence of *Candida albicans* was confirmed in two samples showing 10% occurrence rate (Fig.-2).

Fig.-2: Candida Albicans Isolated from Urinary Samples.

3.3 Study of Anti-Candidal Activity

It has been reported in many cases co-production of varieties of lipoproteins like surfactin/ intruin as well as surfactin/mycosubtilin[13,18].Researches are done to screen and characterize these novel antimicrobial peptides and develop them as a potential alternative in therapeutic and food industries. The antifungal activities of Bacillus strain can be enhanced by manipulating the nutritional requirements. Optimal antifungal activity was obtained when sucrose was used as carbon source followed by glucose. In one such experiment marked increase in surfactin production was observed in *Bacillus subtilis* MZ-7 when sucrose was supplemented[19,20].Addition of fructose, ribose, starch, maltose, glycerol and arabinose increased the microbial activity while mannitol, starch and inorganic carbon sources like $CaCO_3$ did not enhance antifungal activity[21].

In the present study the anticandidal activity of *Bacillus subtilis* was assessed by using different amounts of the antimicrobial extract of *Bacillus subtilis*. Seven different volumes of the cell free extract were poured in the wells made in agar plates seeded with *Candida albicans*. Well 1 having 20 µl and well 2 having 40 µl failed to give any inhibition against the test organism. While well 3 with 70 µl showed the inhibition zone of 2 mm, well 4 having 100 µl showed the zone of 4 mm, well 5 having 200 µl gave a zone of 6mm, well 6 having 400 µl gave a zone of 11mm while well 7 gave the maximum zone of inhibition of 22 mm. The minimum inhibitory concentration sufficient to inhibit the pathogen *Candida albicans* was obtained in 70µl of the extract and the maximum inhibition was given by 700 µl of the extract (Fig.-3).

Fig.-3: Anti Candidal Activity Shown By Bacillus Subtilis.

IV. CONCLUSION

Microbial source has always been an effective alternative for producing anti-fungal agents against the pathogenic fungi and the need for novel sources and their products is an area of research especially in cases of development of drug resistance. *Bacillus subtilis* are potent organisms producing a wide range of antimicrobial substances accounting for antibacterial, antifungal and antiviral activities. Exploring the unexplored complex habitats of ecosystem may end up in obtaining such novel strains and their metabolites. Characterizing these antimicrobial substances would become an effective alternative against multiple organisms. Manipulating their growth conditions would enhance their antifungal activities as well as optimizing their concentrations and further purifications is still an area of research as well as interest.

REFERENCES

[1] A. Nerurkar, P. Solanky, N. Chavda, H. Baria, and B. Desai, Isolation of *Candida* Species in clinical specimens and its virulence factor: The biofilm, 2012.

[2] B. Jha, S. Dey, M. Tamang, M. Joshy, P. Shivananda, and K. Brahmadatan, Characterization of *Candida* species isolated from cases of lower respiratory tract infection, *Kathmandu University medical journal 4(3)*, 2005, 290-294.

[3] Y.M. Clayton, and W. Noble, Observations on the epidemiology of *Candida albicans*, *Journal of clinical pathology*, *19(1)*, 1966, 76.

[4] I. Das, P. Nightingale, M. Patel, and P. Jumaa, Epidemiology, clinical characteristics, and outcome of candidemia: experience in a tertiary referral center in the UK, *International Journal of Infectious Diseases*, *15(11)*, 2011, 759-763.

[5] M. Yilmaz, H. Soran, and Y. Beyatli, Antimicrobial activities of some *Bacillus spp.* strains isolated from the soil, *Microbiological Research*, *161(2)*, 2006, 127-131.

[6] T.R. Klaenhammer, Genetics of bacteriocins produced by lactic acid bacteria, *FEMS microbiology reviews*, *12(1-3)*, 1993, 39-85.

[7] S. Mukherjee, P. Das, and R. Sen, Towards commercial production of microbial surfactants, *Trends in biotechnology*, *24(11)*, 2006, 509-515.

[8] Y.F. Ye, Q. Q. Li, F. Gang, G. Q. Yuan, J.-H. Miao, and L. Wei, Identification of antifungal substance (Iturin A 2) produced by *Bacillus subtilis* B47 and its effect on southern corn leaf blight, *Journal of Integrative Agriculture*, *11(1)*, 2012, 90-99.

[9] K. Arima, A. Kakinuma, and G. Tamura, Surfactin, a crystalline peptidelipid surfactant produced by *Bacillussubtilis*: Isolation, characterization and its inhibition of fibrin clot formation, *Biochemical and biophysical research communications*, *31(3)*, 1968, 488-494.

[10] N. Vanittanakom, W. Loeffler, U. Koch, and G. Jung, Fengycin-A novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3, *The Journal of antibiotics*, *39(7)*, 1986, 888-901.

[11] Y. Hathout, Y. P. Ho, V. Ryzhov, P. Demirev, and C. Fenselau, Kurstakins: A New Class of Lipopeptides Isolated from *Bacillus thuringiensis*, *Journal of natural products*, *63(11)*, 2000, 1492-1496.

[12] N. Roongsawang, J. Thaniyavarn, S. Thaniyavarn, T. Kameyama, M. Haruki, T. Imanaka, M. Morikawa, and S. Kanaya, Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin, *Extremophiles*, *6(6)*, 2002, 499-506.

[13] E.H. Duitman, L.W. Hamoen, M. Rembold, G. Venema, H. Seitz, W. Saenger, F. Bernhard, R. Reinhardt, M. Schmidt, and C. Ullrich, The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase, *Proceedings of the National Academy of Sciences*, *96(23)*, 1999, 13294-13299.

[14] H.P. Bais, R. Fall, and J.M. Vivanco, Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by Pseudomonas syringae is facilitated by biofilm formation and surfactin production, *Plant physiology*, *134(1)*, 2004, 307-319.

[15] Y. L. Yang, Y.A. Ho, H. H. Cheng, M. Ho, and H. J. Lo, Susceptibilites of *Candida* Species to Amphotericin B and Fluconazole The Emergence of Fluconazole Resistance in *Candida tropicalis*, *Infection Control*, *25(01)*, 2004, 60-66.

[16] S. Jaya, and V. Harita, *Candida* Species Isolated from Various Clinical Samples and Their Sus-ceptibility Patterns to Antifungals, *J Med Microbiol*, *1(1)*, 2013, 23.

[17] P. Kim, H. Bai, D. Bai, H. Chae, S. Chung, Y. Kim, R. Park, and Y.T. Chi, Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26, *Journal of applied microbiology*, *97(5)*, 2004, 942-949.

[18] C.C. Huang, T. Ano, and M. Shoda, Nucleotide sequence and characteristics of the gene, lpa-14, responsible for biosynthesis of the lipopeptide antibiotics iturin A and surfactin from *Bacillus subtilis* RB14, *Journal of fermentation and bioengineering*, *76(6)*, 1993, 445-450.

[19] M.M. Al-Ajlani, M.A. Sheikh, Z. Ahmad, and S. Hasnain, Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium, *Microbial cell factories*, *6(1)*, 2007, 17.

[20] S. Joshi, S. Yadav, and A.J. Desai, Application of response-surface methodology to evaluate the optimum medium components for the enhanced production of lichenysin by *Bacillus licheniformis* R2, *Biochemical Engineering Journal*, *41(2)*, 2008, 122-127.

[21] N.M. El-Banna, Effect of carbon source on the antimicrobial activity of *Corynebacterium kutscheri* and *Corynebacterium xerosis*, *African Journal of Biotechnology*, *5(10)*, 2006.