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Phosphatase Production among Candida Species

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ABSTRACT

Several attempts to demonstrate the production of hydrolytic enzyme such as phosphatase among *Candida species* **have been made as, these are the virulence factor that may play important role in the pathogenicity of Candida and also able to attack cell and molecules of host immune system to avoid or resist antimicrobial activity. Phosphatase activity was widely distributed among various species.** *Candida albicans***, which is the most common yeast isolated from clinical material notably produce the enzyme and it is the most consistent, strong, and rapidly active phosphatase-producing organism. Other species also produce phosphatase but in a lesser amount. A group of 33 strains of Candida isolated from infected person were tested for phosphatase activity by using phenolphthalein phosphate as substrate. The visualization of enzyme phosphatase in vitro was conducted in SD broth in which a substrate is added in a culture broth, NaOH & NH_4OH are added according to their respective protocols that were used to visualize the color reaction. As a result different color intensities were observed by species which have an enzyme, while no reaction or color observed by species that do not produce phosphatase. Phosphatase activity among** *Candida species* **was determined by following 3 protocols in which we observed different color intensities, also by plating method in which we observed white precipitation (phosphatase enzyme give precipitation with NaOH and SDA agar).Acid phosphatase was assayed in intact by measuring the liberations of phenolphthalein from Phenolphthalein Diphosphates in terms of O.D at 550 nm. Most of the strains of** *Candida albicans* **were rapidly produce an enzyme phosphatase, while strains other than** *Candida albicans* **including** *C.dubliensis, C.tropicalis, C.parapsilosis, C.kruzei* **and mix growth culture) produces enzyme phosphatase in lesser amount and some give negative results.**

Key words: *Candida species*, Phenolphthalein Diphosphates (PDP), Sabouraud dextrose agar (SDA), Phosphatase, Candidiasis.

INTRODUCTION

The cell wall is essential to nearly every aspect of the biology and pathogenicity of *Candida spp*. The major components of the cell wall are glucan and chitin, which are associated with structural rigidity, and mannoproteins. Among the secreted enzymes are those that are postulated to have substrates within the cell wall and those that find substrates in the extracellular environment. Cell wall proteins have been implicated in adhesion to host tissues and ligands. In addition a number of hydrolytic enzymes have been recovered from both cell-associated locations (cell wall and periplasm) and culture medium whose function is postulated to be within the cell wall. The enzymes are postulated to find substrates and to have their primary function within the cell wall. There are some enzymes whose substrates are not associated with the cell wall but are found in the environment. When hydrolysis of these substrates or action of extracellular proteins

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affects the function and viability of the host, the enzymes may be considered as virulence factors that contribute to the establishment of infection (Chaffin *et al*., 1998). A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group. This action is directly opposite to that of phosphorylases and kinases. Phosphorylation and dephosphorylation usually results in a functional change of the substrate by changing enzyme activity, intracellular location, or association with other proteins. Although hydrolytic enzymes such as acid phosphatase were examined in the given study. Among the catalytic mannoproteins detected outside the plasma membrane barrier in *C. albicans*, acid phosphatase was one of the first to be characterized. It is a candidal hydrolase, for which some role in the pathogenesis of candidiasis has been suggested but not confirmed. *C. albicans* acid phosphatase is an inducible enzyme that has been purified to homogeneity; the purified enzyme is a 125 to 130 kDa mannoprotein with a pH optimum of 3.6 to 4.5. The location (or distribution) within the cell wall structure of acid phosphatase in *C. albicans* is similar to that of acid phosphatase in *S. cerevisiae*, where the enzyme was found to be located in the outermost and innermost cell wall layers. Although the existence of yeast alkaline phosphatase has been described (Schurr & Yagil, 1971; Onishi *et al*., 1979), only the acid phosphatase are secreted and these have recieved the most attention. Acid and alkaline phosphatase has been demonstrated cytological in a variety of bacteria and yeasts.Very little is known about phosphatase activity of other *Candida species* and related yeasts isolated from human clinical specimens (Odds and Hierholzer, 1973). During routine procedures to identify these organisms, studies were also conducted to determine the phosphatase activities of *C. albicans* and other species of yeasts. The enzyme activity was induced by heat and shown to be alkaline phosphatase, such activation resulting in a loss of toxic properties. Several investigators have claimed that certain phosphatase assays on solid media are not sufficiently sensitive and give rise to false negative reaction while other have reported that assays performed in liquid media may give false positive reaction (Cassone, 1989; Rodny *et al*., 1973). Toxic activity may be associated with the activity of hydrolytic enzymes. More recently the phosphatase activity of *Candida species* and other yeasts were evaluated.

Recent studies with intact yeast cells have demonstrated the presence of acid phosphatase in seven pathogenic *Candida species*, in *Cryptococcus neoformans*, and in *Torulopsis glabrata* (in preparation) (Chattaway *et al*., 1971). This may probably suggest the relation between pathogenicity and enzymatic production in some *Candida species*. A micro method is described for the determination of acid and alkaline phosphatase. Phenolphthalein diphosphate is used for substrate and may be prepared by a modified method of Huggins and Taltalay (Fischl *et al*., 1967).

MATERIALS & METHODS

Culture Used: Candida albicans and non *Candida albicans*.

Sample Collected: Department of Microbiology, Jinnah University for Women.

Target for Isolation: To check phosphatase activity among *Candida species*.

Media and Chemical Reagents: Agar (Oxoid), Sabouraud dextrose agar (Merck), dextrose (Oxoid), peptone water (Merck), 0.4% NaOH, 0.01% NaOH (Merck), 0.1%, 1%, 0.01% phenolphthalein diphosphate solution (Fluka Analytical), 0.1 M Tris hydroxyl methyl amino methane (IBI),NaOH 0.4N and 0.1N (Merck), NH_4OH, McFarland standard tubes.

Protocol# 1. For Phosphatase Activity: Phenolphthalein diphosphate was the substrate chosen for the assay. Organisms were suspended in 1ml of 0.1M buffer (Tris hydroxy methyl amino methane)

provide a turbidity equal to McFarland Standard No.3. Add 2ml of 0.1% phenolphthalein diphosphate to suspension & tubes were incubated at 37°C in water bath for 30 minutes. Add 2 to 3 drops of 0.4N NaOH depending on the buffer and it was added to stop the reaction. Tubes were visually observed to determine the extent of phosphatase activity.

Protocol# 2. For Constitutive Phosphatase Activity: Screening test to detect the enzyme activity was conducted in SD Broth. 1% solution of phenolphthalein diphosphate was prepared in distilled water. The substrate was added to 5ml quantities of SD Broth to give final concentration of 0.02% PDP. Yeast stains were inoculated into broth & incubated at 35ºC. After 48 hour, the tubes were mixed on vortex & 1ml was removed & tested for phosphatase activity by adding 1 drop of NH_4OH to 1ml of broth. The color intensity of the reaction ranges from light pink to dark peach.

Protocol#3. For Comparative Quantitative Phosphatase Activity: One set of SD broth with 0.01% phenolphthalein diphosphate (marked as" test") & one set of SD broth without substrate (marked as" control") was inoculated with the culture of *Candida spp*. Incubate at 37ºC for 24 hour. Culture & substrate in tubes incubated at 30-37ºC was determined by spreading sample on SDAgar plate. The remainder of broth containing PDP was centrifuge at 5000 rpm for 10 mins. One ml broth supernatant was removed & mixed with 5ml of 0.1N NaOH. Color intensities were recorded.

RESULTS

A group of 33 different strains of Candida i.e. (*C.albicans, C.dubliensis, C.tropicalis, C.parapsilosis, C.kruzei* and mix growth) isolated from infected patients. The production of phosphatase varies among *Candida species*. The isolated strains were examined for the production of phosphatase enzyme using phenolphthalein diphosphate as substrate. The visualization of enzyme phosphatase in vitro were conducted in SD broth in which a substrate is added in a culture broth, addition of NaOH & NH_4OH with their respective protocols were used to visualize the color reaction. As a result different color intensities were observed by species which have an enzyme, while no reaction or color observed by species that do not produce enzyme phosphatase. Among 33 strains, phosphatase activity was observed in most of the species. Strong phosphatase activity was judged to be (+) (++) (+++), All of the positive strains show light to deep pink (1+, 2+, and 3+) reaction. Each of 11 Candida strains were not producing enzyme phosphatase and were judged to be (-) after 24hours of incubation. Except each of 3 strains showed increase phosphatase production (3+) reaction (Figure 1). In constitutive phosphatase production all of the strains were positive and produce light to dark peach pink (1+, 2+ and 3+) reactions, except 3 strains were negative that do not show any color change (Figure 2). Comparative quantitative assay of phosphatase activity with 33 strains of Candida revealed that *C.albicans* was the most potent phosphatase producing organism tested at 37°C. Phosphatase activity was observed in 30 strains which produce light to intense (1+, 2+,) reaction, except 3 strains were negative and show no color change (Figure 3). Phosphatase activity was not detected in the substrate free broth (as control). In plate methods followed by third protocol white precipitation was examined (phosphatase enzyme give precipitation with NaOH and SDA agar) (Figure 4).

DISCUSSION

By the experiment we examined some strains of Candida give increase amount of phosphatase enzyme while remaining produced small amount of phosphatase enzyme. The acid phosphatase of *C. albicans*, or other yeasts for that matter, appears to be fundamentally different from other *Candida species* with respect to the extra-cellular activity of the enzyme in actively growing cultures. Phenolphthalein diphosphate substrates were used in this study because of their potential colorimetric diagnostic applications. The phosphatase test is not

Table I. Phosphatase Activity by Protocol #1.

S.NO	TUBES	RESULTS ON 1ST DAY	RESULTS AFTER 1 WEEK
1	001	++	+++
2	002	++	+++
3	004	-	++
4	005	++	++
5	006	++	++
6	007	+	++
7	009	+	+++
8	010	-	++
9	011	+	+++
10	012	-	++
11	013	+	++
12	014	+	++
13	015	+	++
14	016	-	++
15	017	+++	+++
16	018	-	+++
17	019	++	++
18	02O	++	+++
19	CSP13	-	++
20	LN3994	-	+
21	CSP24	-	+
22	CSP1567	-	+
23	CT1727	-	-
24	P116	+++	+++
25	E14	+	+
26	A1	+++	+++
27	U8	+	+
28	210	+	++
29	113	+	+
30	501	+	++
31	226	-	-
32	1806	+	+
33	147	+++	+++
34	Control	-	-

Table II. Phosphatase Activity by Protocol #2.

S.NO	TUBES	RESULTS
1	001	++
2	002	+
3	004	++
4	005	+++
5	006	+++
6	007	+++
7	009	+
8	010	+
9	011	+++
10	012	++
11	013	+
12	014	++
13	015	+
14	016	++
15	017	-
16	018	+
17	019	++
18	02O	+
19	CSP13	+++
20	LN3994	-
21	CSP24	+++
22	CSP1567	+++
23	CT1727	+++
24	P116	++
25	E14	-
26	A1	+++
27	U8	-
28	210	+++
29	113	+
30	501	+
31	226	++
32	1806	+++
33	147	+++
34	Control	-

Table III. Phosphatase Activity by Protocol #3.

S.NO	TEST	RESUTLS	OD	CONTROL	RESUTLS	OD
1	001	++	0.075	001	-	0.082
2	002	+	0.080	002	-	0.061
3	004	++	0.095	004	-	0.083
4	005	+	0.022	005	-	0.000
5	006	+	0.044	006	-	0.008
6	007	++	0.083	007	-	0.073
7	009	+	0.030	009	-	0.004
8	010	+	0.087	010	-	0.076
9	011	++	0.101	011	-	0.087
10	012	+	0.071	012	-	0.076
11	013	++	0.058	013	-	0.079
12	014	+	0.041	014	-	0.000
13	015	++	0.006	015	-	0.005
14	016	++	0.031	016	-	0.006
15	017	-	0.072	017	-	0.074
16	018	+	0.038	018	-	0.002
17	019	++	0.010	019	-	0.007
18	020	-	0.046	020	-	0.002
19	CSP13	++	0.171	CSP13	-	0.030
20	LN3994	+	0.077	LN3994	-	0.080
21	CSP24	++	0.145	CSP24	-	0.027
22	CSP1567	++	0.145	CSP1567	-	0.035
23	CT1727	-	0.046	CT1727	-	0.029
24	P116	++	0.186	P116	-	0.043
25	E14	++	0.174	E14	-	0.000
26	A1	+	0.072	A1	-	0.024
27	U8	++	0.148	U8	-	0.028
28	210	+	0.082	210	-	0.087
29	113	++	0.162	113	-	0.035
30	501	++	0.091	501	-	0.074
31	226	++	0.074	226	-	0.083
32	1806	+	0.079	1806	-	0.080
33	147	+	0.075	147	-	0.081

Figure 1

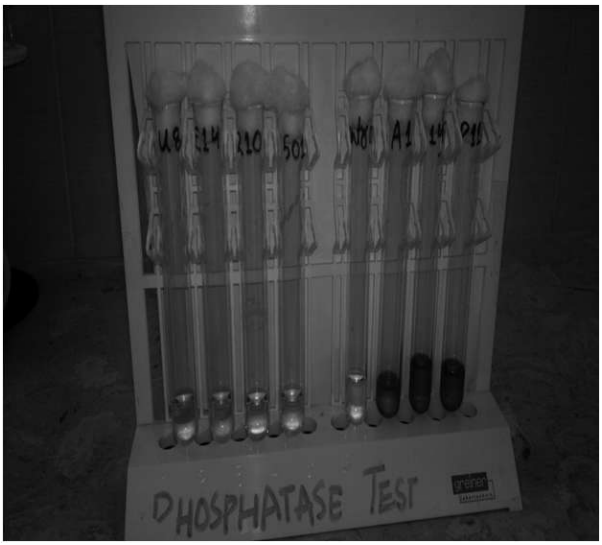

Figure 2

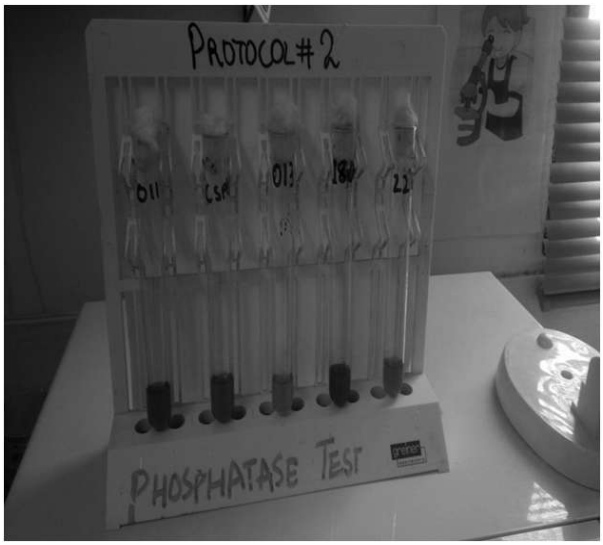

Figure 1. Phosphatase activity was done by protocol#, +ve results were shown by light to deep pink color reactions while -ve results were shown by no color change. Light color reaction seen after 24 hours while, dark color reaction seen after 1 week.

Figure 2. Phosphatase activity was done by protocol#2, +ve results were shown by light to dark peach pink color reactions while -ve results were shown by no color change after 48 hours.

Figure 3

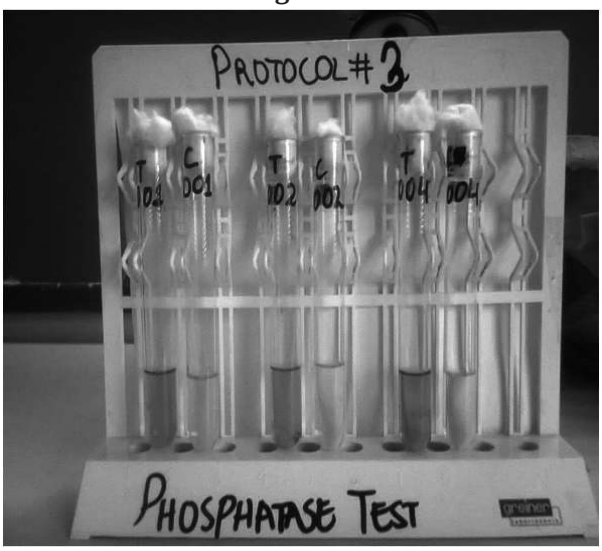

Figure 4

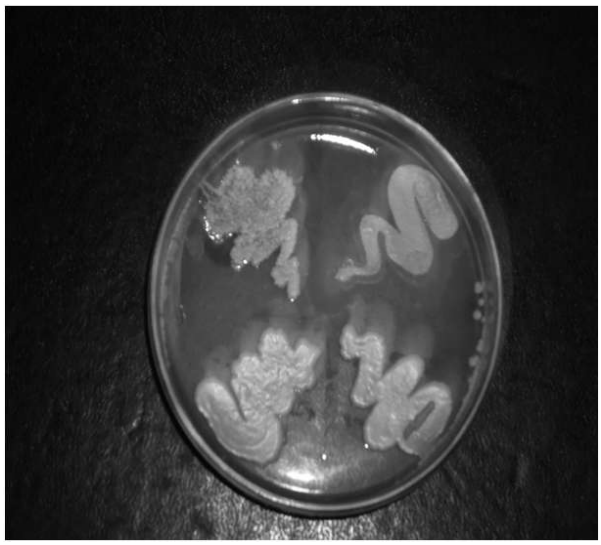

Figure 3. Phosphatase activity was done by protocol#3, +ve results were shown by light to dark peach pink color reactions while –ve results were shown by no color change after 24 hours.

Figure 4. Plate method done by protocol # 3, show white precipitation (phosphates enzyme give precipitation with NaOH on SDA agar).

proposed here as a routinely useful diagnostic test but could be a simple and rapid optional method for the separation of *C. albicans* from other closely related species. Since *Candida species* may cause infection, particularly in the compromised host, the demonstration of acid phosphatase in several yeasts which are not commonly pathogenic in humans may also indicate that such enzymes can not be associated with virulence as in the case of *C. albicans*. The association of phosphatase activity with many clinically significant pathogens i.e. *Candida species* may prove a useful correlate of pathogenicity. This study shows variation in phosphatase production among different *Candida species* depending on several factors such as pH (acid and alkaline), temperature (enzyme may be heat labile), nutrients (enzyme required carbon source for there production), substrate difference (organism may require organic, inorganic and molecular on in ion foam), extra cellular and cell bound enzyme. It may be possible that organism may not have proper nutrient to performed this activity, for example, nature of carbon source seems to play an important role in the regulation of its growth. A fermentable carbon source such as glucose, fructose, dextrose and mannose leads to a constitutive synthesis of the enzyme which stop as soon as the sugar is exhausted, even though the yeast continues the sugar fermentation (Rodney *et al*., 1973).

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