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CHLAMYDOSPORE FORMATION OF *Candida albicans* USING LYOPHILIZED *Eicchornia crassipes* (WATER HYACINTH) AS AN ALTERNATIVE FUNGAL CULTURE MEDIUM

Lorreine Denise W. Castañares

Department of Medical Technology, College of Allied Medical Sciences
Cebu Doctors' University

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Corresponding Author: Lorreine Denise W. Castañares, Department of Medical Technology, College of Allied Medical Sciences, Cebu Doctors' University, Mandaue City, Cebu, Philippines

Abstract: The study evaluates the potential of Water Hyacinth Agar with Polysorbate 80 as a fungal culture medium to stimulate chlamydospore formation of *Candida albicans*. A total of 104 plates were prepared, with 52 plates for the Water Hyacinth Agar as the experimental group and 52 for the Corn Meal Agar as a positive control group. Clinical isolates of *Candida albicans* were streaked onto the plates, incubated at 37°C, and viewed microscopically for the presence of chlamydospores after 24, 48, and 72 hours of incubation. The number of chlamydospores were then counted under the low power objective by three readers. For both groups, the median chlamydospore count increased as the incubation time increased, with the highest median chlamydospore count recorded after 72 hours of incubation. At 24, 48, and 72 hours, the median chlamydospore count of *Candida albicans* was significantly higher in the Corn Meal Agar group compared to that in the Water Hyacinth Agar group because of the higher carbohydrate content in the former. Thus, the presence of the chlamydospores of *Candida albicans* in Water Hyacinth Agar showed that it has the potential to be an alternative fungal culture medium for chlamydospore formation of *Candida albicans*.

Keywords: Candida albicans, chlamydospore, Eicchornia crassipes, fungal culture medium, water hyacinth

I. INTRODUCTION

The global burden of mycoses has been increasing dramatically worldwide. Opportunistic fungal pathogens including *Candida* have worldwide distribution and constitute the majority of invasive fungal infections (Lee & Lau, 2017). Two studies mentioned by Sardi et al. (2013) stated that *Candida albicans* continues to be the

leading cause of Candida bloodstream infections and poses a serious and growing public health problem that includes high mortality rates, increased cost of care and increased length of hospital stay. The primary purpose of this study is to determine the potential of water hyacinth (*Eicchornia crassipes*) with Polysorbate 80 as a fungal culture medium for chlamydospore formation of *Candida albicans*.

Candida albicans can exist in several morphologic forms. The most common is a budding yeast with blastoconidia. *Candida albicans*, when subjected to unfavorable conditions, can be forced to undergo a complex morphologic transformation to form chlamydospores. Chlamydospores are globular, thick-walled asexual spores formed from a hyphal cell (Martin et al., 2005). According to Campanha et al. (2005, Bottcher et al., 2016), chlamydospore identification is frequently used as a reliable and inexpensive diagnostic technique to distinguish *Candida albicans* from other Candida species. Yeast identification procedures usually start with a germ tube test and is followed by carbohydrate fermentation and carbohydrate assimilation tests. These sugar assimilation tests may take 72 hours to 2 weeks and are labor intensive. This results in a delay in the diagnosis and the subsequent prescription of the proper antifungal agent (Baradkar et al., 2010).

Chlamydospore formation on particular media remains the primary method for identifying *Candida albicans* in most medical laboratories (Sukroongreung, 1971). *Candida albicans* can be induced on specialized media to form the thick-walled chlamydospores. The unknown yeast is either streaked onto or cut into this medium in the old standby identification method using Corn Meal Agar. A flamed microscope coverslip is placed on top of the streaked area using the surface inoculation method and then incubated at 35-37^{o}C for 24-72 hours. The petri dish is then placed directly on the microscope stage and examined.

On Corn Meal Agar, only the members of the genus Candida form yeast cells and pseudohyphae. If only yeast cells are visible, then the organism is not a Candida species. If yeast cells and pseudohyphae are seen, the organism is a Candida species. If the organism forms yeast cells, pseudohyphae, and large chlamydospores, then the organism is *Candida albicans*. This test has been used for many years to identify *Candida albicans*. (Bulmer, 1995)

Typical inducing media used for chlamydospore formation should be rich in complex carbon sources. The best-known chlamydospore-inducing media for *Candida albicans* are solid media that contain complex carbohydrates such as Corn Meal Agar (Casal & Linares, 1981, as cited in Citiulo, Moran, Coleman, & Sullivan, 2009).

Low oxygen tension enhances the growth and spore formation of *Candida albicans* and induces the fastest production of abundant chlamydospores (Montazeri & Hedrick, 1984). Chlamydospores can be induced by inoculating *Candida albicans* on rice extract and semisolid agar containing Polysorbate or Tween 80 (1%) to exert surface stress. A polyethylene sheet is overlaid to induce microaerophilic conditions at 30^{o}C (Ingle et al., 2017). Polysorbate 80 (Tween 80, polyoxyethylene sorbitan monooleate) is a fatty acid ester of polyoxyethylene sorbitan. It is often employed in biotherapeutic products as a non-ionic surfactant to avoid surface adsorption and stabilize protein against aggregation caused by stressors like agitation and shear (Shi et al., 2015). Traditionally, chlamydospores have been obtained using a traditional medium for a minimum of 48-72 hours (Alicia, et al., 2006).

Water hyacinth is considered an invasive species that has infiltrated freshwater ecosystems and is considered

one of the worst weeds (Center et al., 1999, as cited in Bhattacharya & Kumar, 2010). Physical, chemical, and biological methods to eliminate water hyacinth are incredibly challenging; throughout the world, a significant amount of money every year is spent on their control (Bhattacharya & Kumar, 2010). According to the Department of Environment and Natural Resources, water hyacinth is deemed a "pest" in the Philippines because of its high productivity of roughly 200 metric tons of dry matter per 10,000 square meter land area under normal conditions (Maulion et al., 2015). However, when viewed from a resource standpoint, water hyacinth appears to be a valuable resource with various distinctive properties. As a result, over the last few decades, research concerning the control and utilization of water hyacinth has been explored extensively (Wang & Wan, 2013). Analysis of dried water hyacinth is comparable to some of the known agricultural residues (Deshpande, Bhotmange, Chakrabarti, & Shastri, 2008). It is 75.8% organic matter, 1.5% N, and 24.2% ash on a zero-moisture basis. Results have shown that its ash content includes 28.7% K2O, 21.0% Cl, 12.8% CaO, 1.8% Na2O, and 7.0% P2O5. Analysis of the plant also showed that it contains, per 100 g, 7.2 g leucine, 4.72 g phenylalanine, 5.34 g lysine, 0.72 g methionine, 4.32 g threonine, 4.32 g isoleucine and 0.27 g valine (Jafari, 2010). Phytochemical analysis by Vasu et al. (2009) showed that the water hyacinth contains 176 mg/g of protein and 195 mg/g of carbohydrates. According to Bhattacharya and Kumar (2010), the cellulose content of water hyacinth is 20%, while the hemicellulose content is 33%. Furthermore, water hyacinth has very high nitrogen content. These provide the source of carbon, nitrogen, and energy necessary for fungal growth.

Numerous studies have been made in the search for different fungal culture media for the isolation of fungi. Most of these use organic materials and support the idea of using locally available organic waste as an alternative fungal culture medium. A study by Akharaiyi and Abiola in 2016 tested the efficacy of using agro-waste in formulating three fungal culture media. The findings of the study showed that all three formulated media supported the growth of the fungi. The results imply that the agro-waste harbors the nutrients necessary for fungal growth, including protein, carbohydrates, and minerals. The protein has high nitrogen content while the carbohydrates serve as a carbon source; both are required for good fungal growth. Sidana and Farooq (2014) developed a sugarcane waste-based fungal culture medium as an alternative to commercially prepared media that are more costly. Fungi need carbon, nitrogen, and an energy source, which the bagasse medium from sugarcane was able to provide and is, therefore, a potential replacement for expensive media in the market. Also, in 2014, Ravimannan, et al. conducted a study to test the different formulations of protein sources, like legume seeds, as an alternative fungal culture medium. Results of the study showed that compared to the commercially prepared control medium, Potato Dextrose Agar (PDA), the test protein media were considered to be good as culture media for fungal studies as these were much cheaper.

Only a few recent studies on alternative fungal culture media used for chlamydospore formation of *Candida albicans* have been made. A study in 1975 by Beheshti et al. used a medium for the sequential production of germ tubes and chlamydospores by *Candida albicans* that was made of "cream of rice" infusion, Tween 80, and ox gall. Results showed that 77 out of 96 cultures of *Candida albicans* produced chlamydospores (80.2%). Most of the cultures were read every day for three days. A similar study by Fleming et al. (1977) described a medium composed of ox gall, Tween 80, caffeic acid (TOC), and Davis agar that allows for the quick presumptive identification of *Candida*

albicans and *Cryptococcus neoformans*. There was an increase in chlamydospore-forming *Candida albicans* strains (97.1 % versus 87.2 %) and a decrease in the time required for chlamydospore development (24 hours versus 48 hours) when compared to Corn Meal Agar control plates. In 2005, Kumar and Menon tested the potential of Tobacco Agar as a new fungal culture medium for the chlamydosporulation of *Candida albicans* and *Candida dubliniensis*. Of the 25 *Candida albicans* tested, 24 (96%) produced chlamydospores after 24 hours of incubation. Light, glucose, and heavy metals have been shown to limit chlamydospore development in Corn Meal Agar. In the Tobacco Agar study, all cultures were exposed to light on the laboratory bench without inhibiting the production of chlamydospores. In a study conducted by Arca et al. (2015), water hyacinth (*Eicchornia crassipes Mart. Solms*), was tested as a fungal culture medium alternative for the cultivation of *Aspergillus niger, Rhizopus sp, Penicillium sp, Candida albicans*, and *Saccharomyces cerevisiae*. Nutrient analysis of water hyacinth at the Industrial Technology Development Institute using the AOAC official method 926.09, 19th ed., 2012 showed that water hyacinth contains phosphorus and nitrogen which are essential minerals for fungal growth and metabolism. In terms of hyphal development, colonial appearance, and morphology, the study indicated that water hyacinth could be a fungal growth medium, but conidia and spore output were lower than with Sabouraud Dextrose Agar.

II. METHODOLOGY

A quantitative true experiment of the chlamydospore formation of *Candida albicans* was performed. The *Candida albicans* clinical isolates were obtained from the Microbiology Department of Cebu Doctors University Hospital. The water hyacinths were acquired from a lagoon in Barangay Pakpakan Road, Lapu-Lapu City. These were washed, dried in the shade, reduced to powder, and soaked in 95% ethanol in the Cebu Doctors' University Medical Technology Laboratory. Concentration of the water hyacinth extract was accomplished using the rotary evaporator in the Cebu Doctors' University Experimental Research Laboratory. Lyophilization of the water hyacinth extract was done at the Chemistry Department Laboratory Facilities at De La Salle University-Manila.

The Corn Meal Agar (Himedia Laboratories) was made by dissolving 17 grams of powder in 1000 mL distilled water and heated to boiling to dissolve the medium completely. Next, 10 mL of Polysorbate 80 (Yana Chemodities) was added. The mixture was autoclaved for 15 minutes at 15 lbs pressure (121°C). After thorough stirring, the mixture was dispensed into sterile Petri plates.

Disease-free samples of water hyacinth *(Eicchornia crassipes Mart. Solms)* were gathered and authenticated at the Department of Agriculture at Mandaue City. The entire plant, except for the roots, was wrapped in cheesecloth and dried under the shade until crisp. The roots were dried at 37°C using a drying oven. A blender was used to pulverize the dried plants and was then immersed in 95% ethanol for 24 hours. After 24 hours, the supernatant was filtered through cheesecloth and filter paper. The final filtrate was processed using a rotary evaporator until a pasty consistency was achieved and then sent for the lyophilization process.

To prepare the Water Hyacinth Agar (WHA), 50 grams of lyophilized water hyacinth, 10 ml of Polysorbate 80 (Yana Chemodities), and 15 grams of bacteriological agar (Bacto agar BD) as a solidifying agent were dissolved in 1000 ml distilled water. It was heated to boiling with frequent agitation and the pH was adjusted to 6.5 utilizing HCl and NaOH. It was then subjected to autoclaving for 15 minutes at 121°C. Approximately 20 ml of slightly

cooled WHA were poured individually into 90 x 15mm Petri dishes. The plates were then allowed to harden at room temperature.

The clinical isolates of *Candida albicans* identified using the VITEK ID-YST system in Cebu Doctors University Hospital were procured. These were subcultured on Sabouraud Dextrose Agar and incubated for 48 hours. A germ tube test was performed for presumptive identification of clinical isolates.

If the germ tube test was positive, the clinical isolates were inoculated by sweeping the colonies on the Sabouraud Dextrose Agar plates using a sterile swab and streaking onto each Water Hyacinth Agar and Corn Meal Agar plate. While holding the inoculating needle at about 45° angle, parallel shallow cuts were made into the agar. A coverslip was then placed on the surface of the agar, covering a portion of the parallel cuts. Plates were incubated at 37ºC, with periodic observations over the next 24, 48, and 72 hours for chlamydospore production. These were examined by placing the plate with its lid on the microscope stage using the low power (100X) objective and counting the number of chlamydospores on the inoculated area covered by the 22 x 22mm coverslip. Using a single-blind experiment, three licensed medical technologists from Cebu Doctors University Hospital did the chlamydospore count on both Corn Meal Agar and Water Hyacinth Agar plates.

A total of 104 plates, with 52 plates per group, were tested. Results were summarized as mean and standard deviation and presented in tables. The non-parametric Mann-Whitney U test was then used to determine the significant median difference between the number of chlamydospores formed by *Candida albicans* in the Water Hyacinth Agar and in the Corn Meal Agar after 24, 48, and 72 hours. Kolmogorov-Smirnov (KS) test indicated that the number of chlamydospores formed by *Candida albicans* in the Water Hyacinth Agar and in the Corn Meal Agar after 24 hours was not normally distributed, D = 0.196, $p < .001$, and D = .123, $p = .048$, respectively. After 48 hours, Kolmogorov-Smirnov (KS) test indicated that the number of chlamydospores formed by *Candida albicans* in the Water Hyacinth Agar was not normally distributed, D = .225, $p < .001$. However, the number of chlamydospores formed by *Candida albicans* in the Corn Meal Agar after 48 hours was normally distributed, D = .114, $p = .087$. After 72 hours, Kolmogorov-Smirnov (KS) test showed that the number of chlamydospores formed by *Candida albicans* in the Water Hyacinth Agar and in the Corn Meal Agar was not normally distributed, D = .201, $p <$ .001 and D = .205, $p = <$ .001, respectively. Hypotheses were tested at a 0.05 level of significance. A p value < .05 alpha level was considered significant. IBM SPSS version 22 was utilized for the processing and analysis of data.

III. RESULTS AND DISCUSSION

Table 1. Number of Chlamydospores of *Candida albicans*

Agar	After 24 hours	After 48 hours	After 72 hours
Water Hyacinth	2.12	2.54	5.73
Corn Meal	13.65	21.87	26.40

The values presented in the table above show that the chlamydospore count of *Candida albicans* in Water Hyacinth Agar is 2.12 after 24 hours, 2.54 after 48 hours, and 5.73 after 72 hours. This shows that the Water Hyacinth Agar could support the growth of *Candida albicans* and induce chlamydospore formation.

Bottcher et al. (2016) states that typical inducing media used for chlamydospore formation are rich in complex carbon sources. Water hyacinth provides this since it contains significant amounts of cellulose (20%) and hemicellulose (33%) (Bhattacharya & Kumar, 2010). It is also shown in the table above that the chlamydospore count increased as the incubation time increased. According to Alicia et al. (2006), the time needed to produce chlamydospores using the standard method is 48-72 hours in Corn Meal Agar. The same was true for the Water Hyacinth Agar, as the chlamydospore count increased the most after 72 hours. This is attributed to the fact that chlamydospores are produced in low-nutrient and low-oxygen environments (Kumar & Menon, 2005). The chlamydospore facilitates the survival of *Candida albicans* under extreme microenvironments by significantly lowering metabolism (Ingle et al., 2017). Thus, the longer the *Candida albicans* was allowed to grow on the Water Hyacinth Agar, the more the medium became nutrient-depleted and the more chlamydospores were formed.

The table above also shows the chlamydospore count of *Candida albicans* in the positive control, Corn Meal Agar. After 24 hours, the median chlamydospore count was 13.65, 21.87 after 48 hours, and 26.40 after 72 hours. Corn Meal Agar is the best-known chlamydospore-inducing medium for *Candida albicans* because it contains complex carbohydrates (Citiulo et al., 2009).

According to Alicia et al. (2006), chlamydospores maintain the standard medium Corn Meal Agar with added Tween 80 in a minimum period of 48-72 hours. The table shows that the mean chlamydospore count of *Candida albicans* increased in Corn Meal Agar the longer it was incubated. The longer the *Candida albicans* grew on the agar, the more the nutrient on the medium was depleted, and the more chlamydospores were formed.

Furthermore, the chlamydospore count of *Candida albicans* in Water Hyacinth Agar had a significant percentage increase after 72 hours of incubation. In contrast, the most significant percentage increase in the chlamydospore count in Corn Meal Agar was after 24 hours of incubation. Chlamydospores are formed by *Candida albicans* when the nutrient source in the culture medium becomes depleted.

In the Water Hyacinth Agar, it took longer for the complex carbon source of cellulose and hemicellulose to be depleted and so the chlamydospore formation increased the most only after 72 hours. This is because cellulose and hemicellulose have glucose repeat units arranged in alternating molecules that are 180 degrees rotated from each other. This arrangement of glucose repeat units is responsible for the strength of cellulose and hemicellulose which was why it took longer for them to be broken down into carbon and nitrogen as nutrient source for fungal growth.

In the Corn Meal Agar, the complex carbon source was depleted faster and so the increase in chlamydospore count was after only 24 hours of incubation. Starch, as the complex carbon source in Corn Meal Agar, has glucose repeat units that are linked together in the same orientation making it weaker in structure and easily broken down to carbon and nitrogen.

Table 2. Mann-Whitney U Test for Number of Chlamydospores of *Candida albicans*

Time	U value	*p* value	Conclusion
After 24 hours	< .0001	< .001	There is a significant difference between groups

After 48 hours	< .0001	< .001	There is a significant difference between groups
After 72 hours	< .0001	< .001	There is a significant difference between groups

In Table 2, the difference in the median chlamydospore count of *Candida albicans* between Water hyacinth agar and Corn Meal Agar after 24, 48, and 72 hours was computed to determine if the medians of the experimental group and the positive control group differed significantly. As seen in the table, there was a significant difference between the two groups. This significant difference meant that at 24, 48, and 72 hours of incubation, *Candida albicans* produced a significantly smaller number of chlamydospores in the Water Hyacinth Agar than in the positive control Corn Meal Agar.

This indicates that although the Water Hyacinth Agar induced chlamydospore formation of *Candida albicans*, the chlamydospore count was incomparable to that of the Corn Meal Agar. This can be explained by the carbohydrate content of Water Hyacinth Agar which is significantly lower than that of Corn Meal Agar. Nevertheless, the presence of chlamydospores indicates the growth of *Candida albicans* despite the smaller number of chlamydospores in the Water Hyacinth Agar. Moreover, the fact that chlamydospores were able to form in the Water Hyacinth Agar indicates that it could be a fungal culture medium for chlamydospore formation of *Candida albicans*.

IV. CONCLUSION

Lyophilized *Eicchornia crassipes* (water hyacinth) with Polysorbate 80 has potential for a culture medium for chlamydospore formation of *Candida albicans*.

References

Akharaiyi F.C. & Abiola, M.A.(2016). Isolation and cultivation of fungi with agrowastes formulated media. *Der Pharma Chemica*, *8*(9), 56-62 https://www.derpharmachemica.com/pharma-chemica/isolation-and-cultivation-of-fungi-with-agrowastes-formulated-media.pdf

.

Arca, A.B., Zamora M.R., Baybayon, R.A., Espeleta, K.B., Onrada, S.J., Yutuc, P.A., & Nuevo, J.J. (2015). Evaluation of water hyacinth (*Eicchornia crassipes Mart. Solms*) as an alternative fungal culture medium. *Asia Pacific Journal of Medical Laboratory Science*, *3*(2015), 35-42.

Beheshti, F., Smith, A., & Krause, G. (1975). Germ tube and chlamydospore formation by *Candida albicans* on a new medium. *Journal of Clinical Microbiology*, *2*(4), 345-348. http://jcm.asm.org/content/2/4/345.full.pdf

Bhattacharya, A. & Kumar, P. (2010). Water hyacinth as a potential biofuel crop. *Electronic Journal of Environmental, Agricultural and Food Chemistry, 9*(1), 112-122. https://www.cabi.org/ISC/FullTextPDF/2010/20103065885.pdf

Bottcher, B., Pollath, C., Staib, P., Hube, B., & Brunke, S. (2016). Candida species rewired hyphae developmental programs for chlamydospore formation. *Frontiers in Medical Microbiology, 7.* https://doi.org/10.3389/fmicb.2016.01697

Bulmer, G. (1995). Fungus diseases in the orient (3rd ed.). Manila, Philippines: Rex Book Store Inc.

Citiulo, F., Moran, G., Coleman, D., & Sullivan, D. (2009). Purification and germination of *Candida albicans* and *Candida dubliniensis* chlamydospores cultured in liquid media. *Federation of European Microbiological Societies FEMS Yeast Research, 9*(7), 1051–1060. https://doi.org/10.1111/j.1567-1364.2009.00533.x

Deshpande, S.K., Bhotmange, M.G., Chakrabarti, T., & Shastri, P.N. (2008). Production of cellulase and xylanase by *Trichoderma reesei* (QM 9414 mutant), *Aspergillus niger* and mixed culture by solid-state fermentation (SSF) of water hyacinth (*Eicchornia crassipes*). *Indian Journal of Chemical Technology, 15*, 449-456. https://www.semanticscholar.org/paper/Production-of-cellulase-and-xylanase-by-Trichoderma-Deshpande-Bhotmange/9d9e7bc8691bc96778ca011db969b79e7ee1349a

Fleming, W. H., Hopkins, J. M., & Land, G. A. (1977). New culture medium for the presumptive identification of *Candida albicans* and *Cryptococcus neoformans*. *Journal of Clinical Microbiology*, *5*(2), 236–243. http://jcm.asm.org/content/5/2/236.full.pdf

Ingle, S., Kodgire, S., Shiradhone, A., Patil, R., & Zore, G. (2017). Chlamydospore specific proteins of *Candida albicans*. *Data*, *2*(3), 26. https://doi.org/10.3390/data2030026

Jafari, N. (2010). Ecological and socio-economic utilization of water hyacinth (*Eichhornia crassipes Mart Solms*). *Journal of Applied Sciences and Environmental Management, 14*(2), 43 – 49. https://doi.org/10.4314/jasem.v14i2.57834

Jayanthi, P. & Lalitha, P.(2012). Comparison Of conventional and sound assisted methods for extraction of *Eichhornia Crassipes(Mart.) Solms. Asian Journal of Pharmaceutical and Clinical Research, 6*(1), 143-146. http://www.ajpcr.com/Vol6Issue1/1523.pdf

Kumar, C.P.G. & Menon, T. (2005). Tobacco agar: a new medium for chlamydosporulation in *Candida albicans* and *Candida dubliniensis. Medical Mycology*, *43*(5), 473-475. https://doi.org/10.1080/13693780400029205

Lee, P. P., & Lau, Y.-L. (2017). Cellular and molecular defects underlying invasive fungal infections—revelations from endemic mycoses. Frontiers in Immunology, 8. https://doi.org/10.3389/fimmu.2017.00735

Martin, S., Douglas, L., & Konopka, J. (2005). Cell cycle dynamics and quorum sensing in *Candida albicans* chlamydospores are distinct from budding and hyphal growth. *Eukaryotic Cell, 4*(7), 1191–1202.https://doi.org/10.1128/EC.4.7.1191-1202.2005

Mayer, F., Wilson, D., & Hube, B. (2013). Candida albicans pathogenicity mechanisms. *Virulence* *4*(2), 119–128. https://doi.org/10.4161/viru.22913

Maulion, R., Hiwatig, K., Rendon, C.J., & Torrano, E.M. (2015). Utilization of water hyacinth (*Eichhornia crassipes*) for phytoremediation of hexavalent chromium in simulated

wastewater. *Asia Pacific Journal of Multidisciplinary Research, 3*(4), 117-123. www.apjmr.com/wp-content/uploads/2015/12/APJMR-2015-3.4.4.18.pdf

Montazeri, M. & Hedrick, H.G. (1984).Factors affecting spore formation of a *Candida albicans* strain. *Applied Environmental Microbiology, 47*(6), 1341-1342. http://aem.asm.org/content/47/6/1341.full.pdf+html

Ravimannan N., Arulanantham, R., Pathmanathan, S., & Niranjan, K. (2014). Alternative culture media for fungal growth using different formulation of protein sources. *Annals of Biological Research, 5*(1), 36-39. http://scholarsresearchlibrary.com/archive.html

Ryan, K.J. (2010). Pathogenic fungi. In K.J.Ryan, C.G.Ray, N.Ahmad, W.L.Drew, & J.J.Piorde (Eds.), *Sherris Medical Microbiology : An Introduction to Infectious Diseases* (5th ed.). Retrieved December 4, 2017, from http://lalashan.mcmaster.ca/theobio/projects/images/c/c0/An_Introduction_to_Infectious_Diseases.pdf

Sardi, J.C.O., Scorzoni, L., Bernardi, T., Fusco – Almeida, A.M., & Giannini, M.J.S. (2013). Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of Medical Microbiology, 62*(Pt.1), 10–24. https://doi.org/10.1099/jmm.0.045054-0

Shi,S., Chen,Z.,Rizzo,J., Semple,A., Mittal,S., Cheung,J.,…Shameem, M. (2015). A highly sensitive method for the quantitation of polysorbate 20 and 80 to study the compatibility between polysorbates and m-cresol in the peptide formulation. *Journal of Analytical & Bioanalytical Techniques, 6*(3), 1-8. https://doi.org/doi:10.4172/2155-9872.1000245

Sidana, A. & Farooq, U. (2014). Sugarcane bagasse: a potential medium for fungal cultures. *Chinese Journal of Biology*, *2014*(2014), 1-5. https://doi.org/10.1155/2014/840505

Vasu, K., Goud, J.V., Suryam, A., & Singara Charya, M.A. (2009). Biomolecular and phytochemical analyses of three aquatic angiosperms. *African Journal of Microbiology Research, 3*(8), 418-421. https://academicjournals.org/article/article1380277547_kandukuri%20et%20al.pdf

Wang Z. & Wan J. (2013). *An economic analysis of the use of water hyacinth for phytoremediation and biogas production in Dianchi Lake, China. WorldFish (ICLARM)* https://www.semanticscholar.org/paper/An-Economic-Analysis-of-the-Use-of-Water-Hyacinth-Wang-Wan/437d75da116935a356d9ad208ec85d1a42a9cdee