Understanding the role of GlcNAc in *Candida albicans* by metabolic and transcriptional analysis

Thesis Submitted for the Award of the Degree of

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in

Biochemistry

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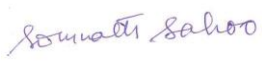
2023

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Understanding the role of GlcNAc in *Candida albicans* by metabolic and transcriptional analysis**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision **Dr. Sarika Sharma**, working as **Professor**, in the Department of **Microbiology** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**Understanding the role of GlcNAc in *Candida albicans* by metabolic and transcriptional analysis**" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the **Biochemistry**, is a research work carried out by **Somnath Sahoo, (Registration No. 11919248)**, is bonafide record of his original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

The yeast that lives in the human gut "*Candida albicans"* is pathogenic and causes a range of mucosal and deep tissue invasions in immunocompromised hosts. Candida demonstrates considerable "metabolic flexibility" and "dynamic morphogenetic" change to survive and establish virulence in host niche environments. Apart from functioning as an excellent carbon and nitrogen source, the amino sugar "N-acetylglucosamine (GlcNAc)" found at host infection sites also drives cellular activation in this pathogen. GlcNAc has several functions in *Candida albicans*, including metabolism, scavenging, import, morphogenetic adaptation ("yeast-hyphae" and "white-opaque" phenotypic switch), virulence, and "GlcNAc-induced cell death (GICD)". Many investigations have centered on determining the molecular mechanisms associated with GlcNAc-induced cellular activities. The contemporary study concentrated on GlcNAc-induced metabolic alterations linked to phenotypic changes. We used gas chromatography-mass spectroscopy (GCMS) and LC-MS, high-throughput and sensitive method, to reveal global metabolomics alterations that occur in Candida cells grown under GlcNAc versus glucose conditions. High-resolution "field emission scanning electron microscopy" (FE-SEM) was used to examine the morphogenetic transition connected with metabolic alterations. The RNA levels of virulence and hyphal-specific genes of induced cells were also evaluated by real time-Reverse Transcriptase-PCR (RT-RT-PCR). Metabolite study indicated that metabolites involved in the "glyoxylate pathway", "oxidative metabolism", and "fatty acid catabolism" were upregulated, which likely aided in the production of GlcNAc-induced hypha-specific components. GlcNAc-grown cells were also marginally more sensitive to "amphotericin B" treatment. These findings add to our understanding of the development of antifungal medicines for the cure of candidiasis in people.

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1. Introduction

1.1 "*Candida albicans*"*:*

The most well-known and widespread human fungal diseases are caused by "*Candida albicans."* It is an opportunistic dynamic fungal pathogen. It inhabits the mammalian gastrointestinal tract as well as other mucosal surfaces primarily as a generous, commensal organism, but causes a variety of mucosal and deep tissue diseases in immunocompromised individuals or when a large contagious inoculum is presented. *Candida* is the fourth most common cause of nosocomial infection over half of which can be fatal [1]. At least 70% of healthy persons have "commensal *C. albicans*" in their skin, mouth, gastrointestinal tract, and female reproductive tract [2, 3, 4, 5]. *C. albicans* and several closely related species have the ability to participate in "parasex". There are two pairing classes in *C. albicans*—a and alpha—as well as orthologs of numerous genes important for meiosis in *S. cerevisiae* and additional eukaryotes. A tiny percentage of clinical isolates are homozygous for the "mating-type locus", indicating that they may be able to reproduce. Diploid or nearly diploid clinical isolates predominate. To create tetraploids through breeding, a physiological/epigenetic transition from the typical "white" phase to a "opaque" condition is required. *Candida* species are fungi that develop as yeasts and are "imperfect," meaning they appear to lack a full sexual cycle. Additionally, the budding "yeast" and "pseudohyphal" (elongated form) cells found in other *Candida* species and *C. albicans*, which is believed to be an "obligate diploid", can also produce genuine filamentous hyphae. Ironically, the availability of contemporary medical therapies like "cancer chemotherapy," "organ transplantation" and overuse of antibiotics has increased the number of people who are susceptible to *C. albicans* infection.

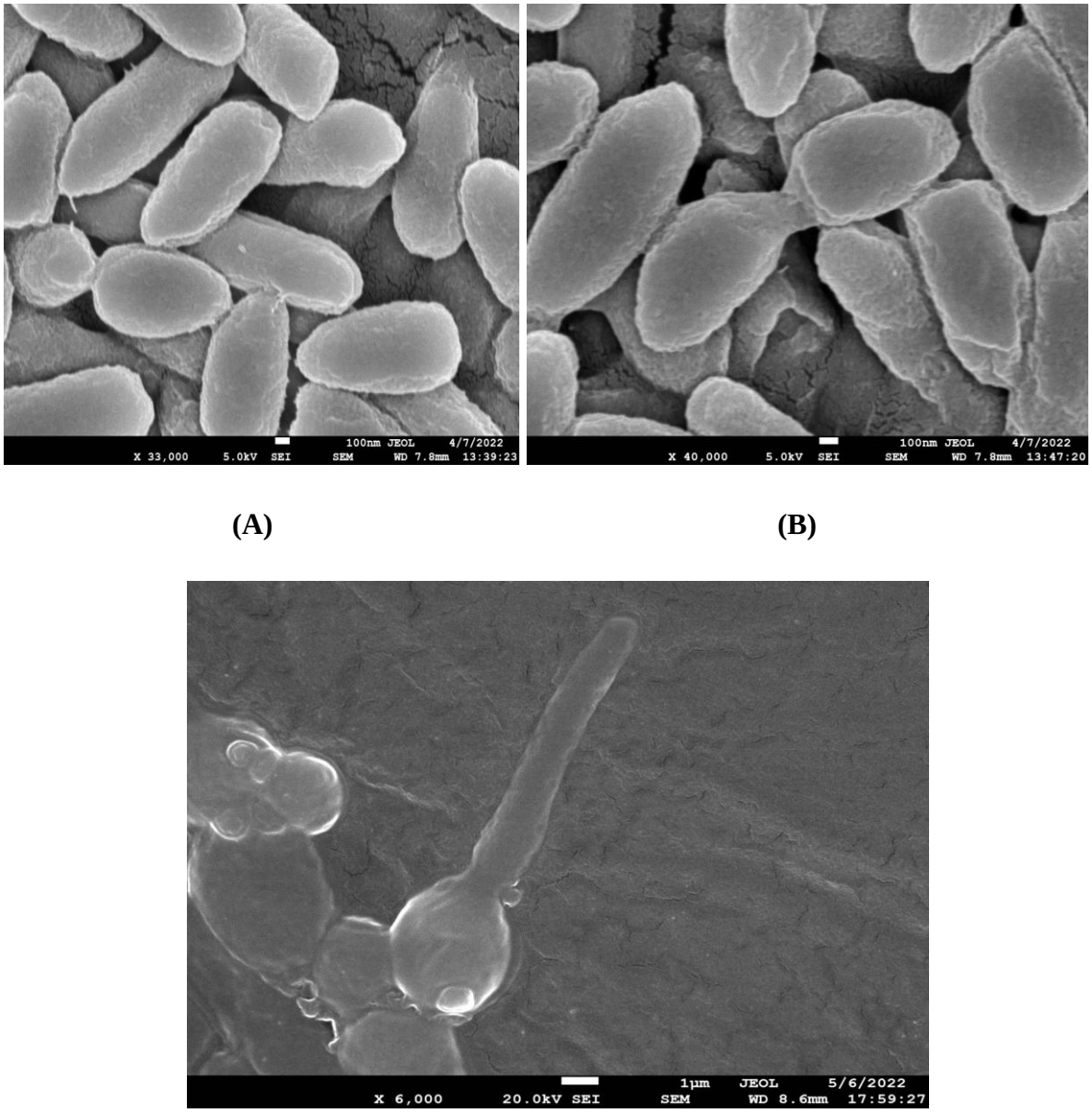
1.2 Morphological Transformation:

The fungus kingdom has a high degree of phenotypic flexibility. Many fungus species exhibit morphological changes in response to natural signals. *C. albicans*' morphological pliability is a pivotal virulence factor [183]. Contrary to dimorphic fungal infections of humans (such as *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Penicillium marneffei*) [6], *C. albicans* is found in dual forms "yeast and filamentous" inside the host and may undergo reversible transformations among yeast, pseudohyphal, and hyphal development forms. [7, 8]. Other than these three forms there are also different conventional *Candida albicans* cell types

like chlamydospores and yeast-like morphotypes such as grey, “opaque(a/α),” and “gastrointestinally induced transition” (GUT) cells. The morphology, mechanism of division, occurrence, and pathogenicity potential of these cell types varies. We emphasize increasing understanding regarding the interelation of these various morphotypes with different host habitats and proclivity for “virulence vs commensalism.” Eventually, we looked at the environmental factors, “signalling routes,” and “transcriptional regulatory chains” that influence morphological transformations. The hyphal form is predominant in the infection procedure because it advances tissue penetration and immune cell evasion [9, 10]. Morphological changes are hypothesized to aid pathogenicity by allowing tiny budding cells to disperse in the circulation and lengthy hyphal filaments to encourage penetration into tissues and biofilm formation [11]

1.3 Yeasts, pseudohyphae, hyphae, and chlamydospores:

Yeasts reproduce through “budding,” and “nuclear division” takes place at the junction of the mother and descendant cells. Because offspring cells are entirely separate from their mother cells following cytokinesis, yeasts are called unicellular. In contrast, “hyphal cells” are more like fine tube-mold cells. The nuclear division takes place within hyphal daughter cells, and a progeny nucleus migrates backwards into the mother cells [12, 13]. Following cytokinesis, hyphal cells stay firmly linked end-to-end, resulting in multicellular, sparsely branching, filamentous formations known as mycelia. Ellipsoid-form “pseudo hyphal” cells exhibit characteristics of both the forms “yeasts and hyphae,” and it is unclear whether they are true cell types or an intermediary between the better-distinguished cell types [14]. No “in vitro” conditions are known to produce pure, persistent populations of “pseudohyphae,” in contrast to yeasts and hyphae. In contrast to “hyphae,” junctions are defined by visible marks or indents. “Chlamydospores” are huge, globular, wide-walled cells seen “in vitro” under extreme circumstances like malnutrition and hypoxia [15]. “Suspensor cells,” which are found at the far-end extremities of “mycelial filaments,” generate chlamydospores. Inside the suspensor cell parent, nuclear division occurs, accompanied by the transfer of an offspring nucleus to the emerging “chlamydospore,” which stays connected to its mother cell [16].



(A) **(B)**

(C)

Figure 1: Morphological forms of *Candida albicans*: (A) Yeasts (B) Psedohyphae (C) Hyphae

1.4 "White–opaque" switching and Mating:

"Candida albicans," human commensal yeast, experiences an epigenetic flip between two different kinds of cells known as "white and opaque." Soll and colleagues originally identified white opaque flipping over a decade ago [17], and it is only seen in a few detached isolates of *Candida albicans* reviewed [18]. White-phase cells are spherical, white, and form dome-moulded colonies on solid agar plates, and express a group of "white-specific genes." Whereas, "opaque cells" are bigger and longer, their colonies seem dimmed and flatten against the agar, have more pronounced vacuoles [19] and they express a collection of "opaque-specific genes."

The metabolic states, mating habits, preferred host habitats, interactions with the "host immune system," cell and colony morphologies of these two cell types are only a few of the many differences between them. Environmental variables can have a significant impact on switching rate [20]. "White and opaque" cell types exhibit unique features, which are mostly due to the contrasting regulation of roughly 400 genes, or concerning 7% of the genome [21; 22]. Significant variations exist between white and opaque cells in metabolic preferences [21], environmental reactions [23], biofilm interactions [24, 25], and the capacity to pair [26]. Cell surface 'pimples' (protuberances with an unclear biological purpose discovered by scanning electron microscopy) are another opaque (a or α)-specific characteristic [11], susceptibility to different filamentation-inducing cues [27, 28] relative resistance of host neutrophils and macrophages to phagocytosis [25, 29]. It is critical in the pathogen-host connection.

The "transcriptional regulatory proteins" expressed by the *Candida albicans* "mating-type" (MTL) locus affect the capacity of *Candida albicans* to undergo "white-opaque switching." MTL regulators that govern and suppress "white-opaque switching" are two homeodomain proteins, Mtla1 and Mtlα 2. It is shown that "mating-type locus homeodomain" proteins govern both "white-opaque switching" and "sexual mating". "Opaque cells" mate about 10^6 times more effectively than "white cell" forms. These findings indicate that the pathogen *Candida albicans* experiences a "white-to-opaque" transformation as a crucial stage in the mating process and that opaque cells represent a type of cell that is capable of mating. Because "white cells" are more resilient than "opaque cells" in a mammalian host, which permits the organism to withstand the rigors of life while producing mating-competent cells. An "**a**" cell must come into contact with a "**α**" cell in order to reproduce, however mating can only take place when both cells are opaque.

What causes the "white-to-opaque" transition? It has been hypothesized that the circuit becomes energized when constituents of the "switching circuit" attain a censorious threshold concentration. WOR1, the first key regulator of the "opaque state" lies at the heart of this circuit [30, 31, 32]. "WOR1" expression creates a direct "positive feedback" cycle by attaching to its own promoter and switching on its own transcription. As this feedback loop is activated, "WOR1" transcript measure in opaque cells rise 40-fold when contrasted to white cells. The a1-α2 heterodimer actively represses the WOR1 promoter, revealing why "a/α" cells cannot transform from "white to opaque." When inadequate levels of the regulators are transmitted onto daughter cells, the reverse process, opaque-to-white switching, occurs, and the circuit shuts down [25]. The established regulatory circuit is completed by three more transcriptional

regulators WOR2, EFG1, and CZF1 [33, 34, 35]. The circuit is generally idle in the “white state,” which is the default condition, based on this model. When the circuit becomes active, switching occurs; due to the succession of “positive feedback” loops, the transitional circuit can remain stimulated for several generations.

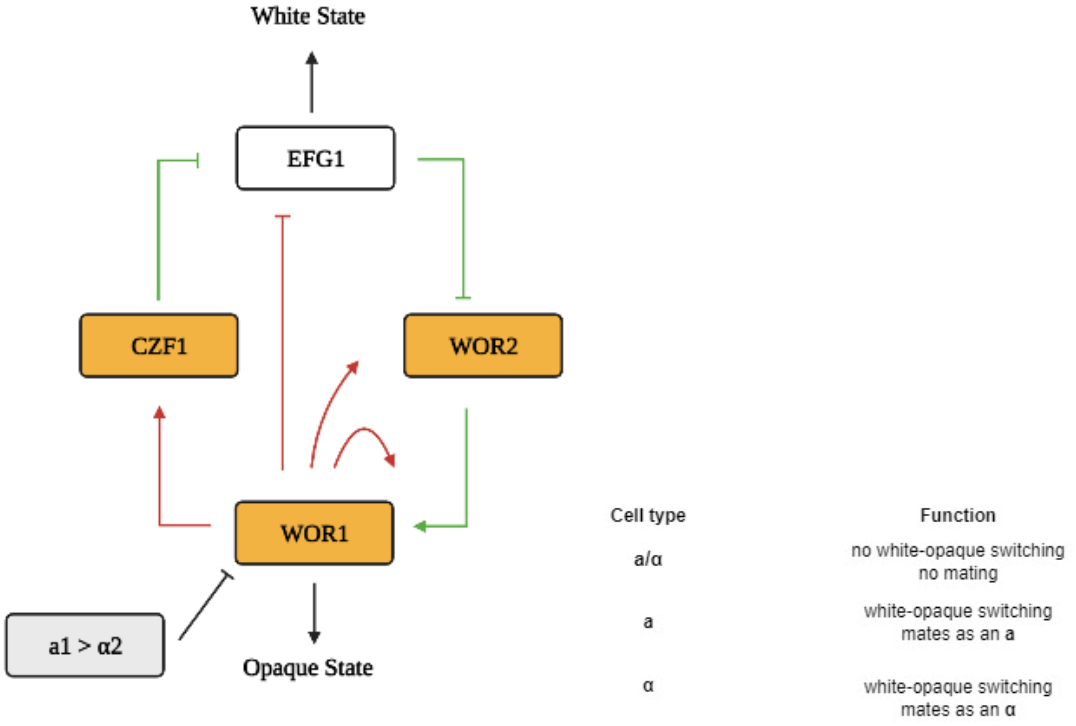


Figure 2: Genes enhanced in the white and opaque forms, respectively, are represented by a white and yellow circular box. Relationships founded on DNA epistasis are represented by green lines. According to the enrichment of Wor1 in chromatin immunoprecipitation studies, red lines show the Wor1 regulation of each gene. Based on each gene's white and opaque state expression, activation (arrowhead) and suppression (bar) are deduced [33].

1.5 Virulence due to morphogenetic transition:

On solid media, hyphae are inherently invasive. Hyphal tip cells display “thigmotropism,” or the remarkable capacity to 'follow' over “substrate surface” irregularities. Furthermore, hyphae express various virulence factors specific to certain cell types like adhesins (hyphal wall protein 1 (Hwp1), Als10, factor activated 2 (Fav2), agglutinin-like protein 3 (Als3), and Pga55), “antioxidant defence proteins” like “Superoxide dismutase 5” (Sod5), “tissue-degrading

enzymes" including "secreted aspartyl protease" (Sap4, 5, 6), and a recently discovered "cytolytic peptide toxin" like "extent of cell elongation protein 1" (Ece1) [36, 37, 38, 39]. In models of superficial candidiasis, such as oropharyngeal [40, 41] and vulvovaginal [42] infection, the enhanced pathogenicity potential of hyphae has been thoroughly demonstrated. Through a particular connection linking the "hyphal adhesin," "Als3," and the host "epithelial cadherin (E-cadherin)", hyphae stimulate endocytic absorption by cultured "human oral epithelial cells"; ingested hyphae subsequently continue to harm the host cells [41]. "Hyphae" can also actively infiltrate "oral epithelial cells," probably by physical coercion and enzymes secreted [9, 43]. As a result, in a reconstructed model of "human oral epithelial tissue," hyphae activate many pro-inflammatory "signalling pathways" in the host, but yeasts, which colonise the "epithelium's surface" without causing harm, activate a more masked "inflammatory response" [40]. Both cell types appear to respond to candidiasis. "Yeasts, hyphae, and pseudohyphae," were all found in clinical specimens retrieved from both human and animal with widespread candidiasis [44, 45, 46]. These findings underscore the importance of yeast-pseudohyphae-hypha morphogenesis in "*C. albicans"* host interactions and propose that yeasts may play various roles in distinct host habitats. Unlike the other types of cells, "chlamydospores," which are easily produced in vitro [47; 48] have seldom been found in clinical isolates or diseased "animal models" [11], and their biological relevance is unknown [49].

1.6 Sugar sensing and utilization to survive in the host niche:

As *C. albicans* adjust their metabolism based on the nutrients available, they may cause infections at various host locations. Both "nutrient-rich" and "nutrient-poor" environments allow them to multiply. It is because of this adaptability that these fungi are effective pathogens. Sugars are vital nutrients, and several "sugar-sensing systems" must exist since the amount of sugar varies based on the host niche. Several virulence traits of these fungal diseases, including "adhesion, oxidative stress resistance, biofilm formation, morphogenesis, invasion, and tolerance to antifungal drugs," depend on their ability to sense sugars.

Since glucose is the most prevalent hexose sugar on the planet, many organisms chose it as their primary carbon source. Because glucose is essential to metabolism, it serves as a substrate for transporters as well as a ligand for receptors. Consequently, glucose acts as a precursor for the creation of other biomolecules and for the fermentation or respiration processes that provide

energy. In *C. albicans*, "hexose transporter" gene induction occurs because of CaHgt4 detecting glucose. It is crucial to remember that other sugars and sugar analogs—such as N-acetylglucosamine in the case of *C. albicans*—as well as glucose are significant carbon sources. It has also been studied that *C. albicans* cells utilizing glucose are commensal and non-pathogenic whereas the cells that take up N-acetylglucosamine (GlcNAc) as a carbon source turn pathogenic or virulent [56]. To know the relation between their metabolic flexibility and virulence, we need to study *C. albicans* induced in different sugars mentioned above.

1.7 GlcNAc and its multifaceted role in *Candida albicans*:

The capacity to use different carbon derivation and yeast-hyphal morphogenetic plasticity is important for pathogen viability, host niche colonization, and virulence of *Candida albicans* [50, 51, 52, 53]. "N-acetylglucosamine (GlcNAc)" is a common carbon source in the environment, as it is found in fungal chitin, "bacterial cell wall" peptidoglycan, "insect exoskeleton," and mammalian extracellular matrix [54, 55]. The finding demonstrates that "N-acetylglucosamine (GlcNAc)" is not only a potentially superior carbon and nitrogen supplier [50, 56], but also an efficacious inducer of signalling to brace the articulation of its "catabolic genes" [57] and "morphogenetic transitions," which includes "yeast to hyphal" alteration [58, 59, 60], "white opaque switching", and an "epigenetic transformation" that directs the mating procedure in *Candida albicans* [61]. GlcNAc-induced cell death (GICD) [62] is an occurrence marked by the generation of "reactive oxygen species (ROS)", accompanied by brisk cell death using both "apoptotic and necrotic" process, and is most likely caused by GlcNAc-induced activation of key cellular processes. Given the sugar's multifaceted role, there is an urgent need to comprehend the "GlcNAc-triggered molecular pathway" of sensing, utilization, and signalling, as well as the numerous "transcription factors" and proteins associated with this activity.

1.8 Different metabolism pathways of GlcNAc in "*Candida albicans*":

Internalized GlcNAc can directly induce signalling by binding to the "GlcNAc sensor (Ngs1)" via the "GlcNAc-specific transporter (Ngt1)". GlcNAc is metabolized by GlcNAc kinase (Hxk1) to GlcNAc-6-P, which is again catabolized by sequential "Deacetylase (Dac1)" to form Glucosamine-6-p and "Deaminase (Nag1)" to produce glucose-6-phosphate, which provides energy or transformed to "UDP-GlcNAc", which is associated with anabolic

processes [183]. Internally synthesized "GlcNAc-6-P" is used in anabolic functions like "chitin synthesis", GPI-anchors, and "N-glycosylation," but it is not involved in the "GlcNAc signalling pathway". GlcNAc-6-P is synthesized from fructose-6-P via the consecutive action of "GlcN-6-P synthase (Gfa1)" and "GlcNAc-6-P acetyltransferase (Gna1)" (Glycolysis). The activity of "phosphor acetylglucosamine mutase" (Agm1) and "UDP GlcNAc pyrophosphorylase" is to convert GlcNAc-6-P to UDP-GlcNAc (Uap1) [56].

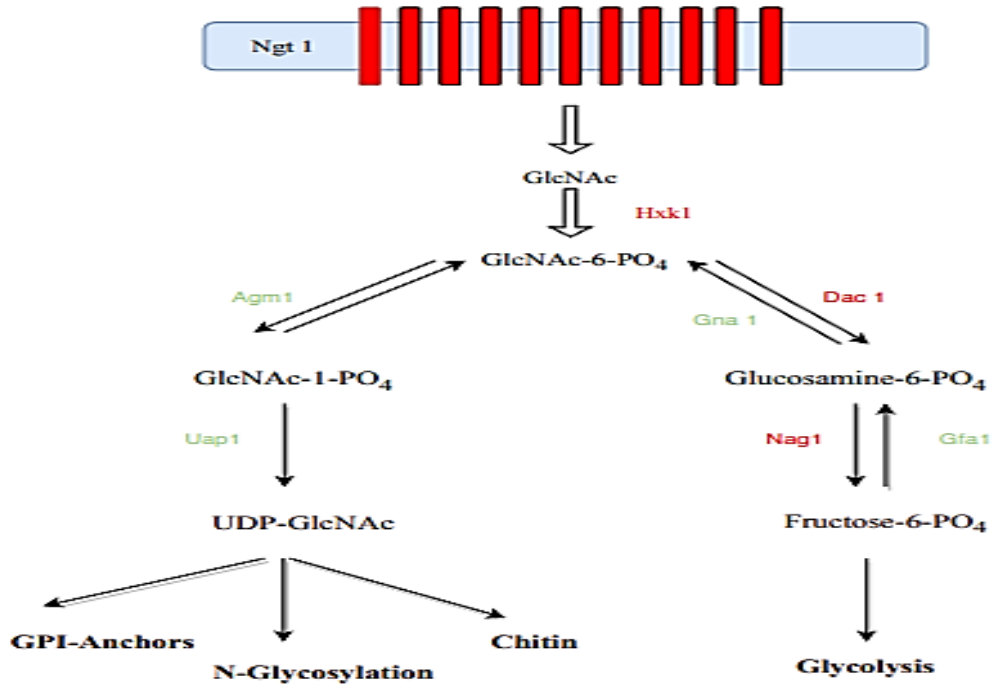


Figure 3: Metabolism of N-Acetylglucosamine (GlcNAc) by *C. albicans*. NGT1 acts as a transporter that uses extracellular GlcNAc. "HXK1" is a kinase that converts these GlcNAc to "GlcNAc-6-phosphate". "DAC1" is a deacetylase which transforms "GlcNAc-6-phosphate to Glucosamine-6-phosphate". "NAG1" acts as a deaminase that converts the product to Fructose-6-phosphate and leads to glycolysis. The anabolism process occurs for GPI-Anchors, N-glycosylation, and chitin formation [56].

1.9 GlcNAc Signailing pathway in *Candida albicans*:

Studies using a "*C. albicans* deletion mutant library" aided in the discovery of "GlcNAc sensor" "Ngs1", that transduces signals simply by specific obligatory to free "GlcNAc" to stimulate a transcriptional reaction within the nucleus [63]. This discovery has aided in the understanding of the molecular process of "GlcNAc signalling" to regulate the expression of genes implicated in GlcNAc catabolism and "GlcNAc-induced filamentation" [63]. Ngs1 uses

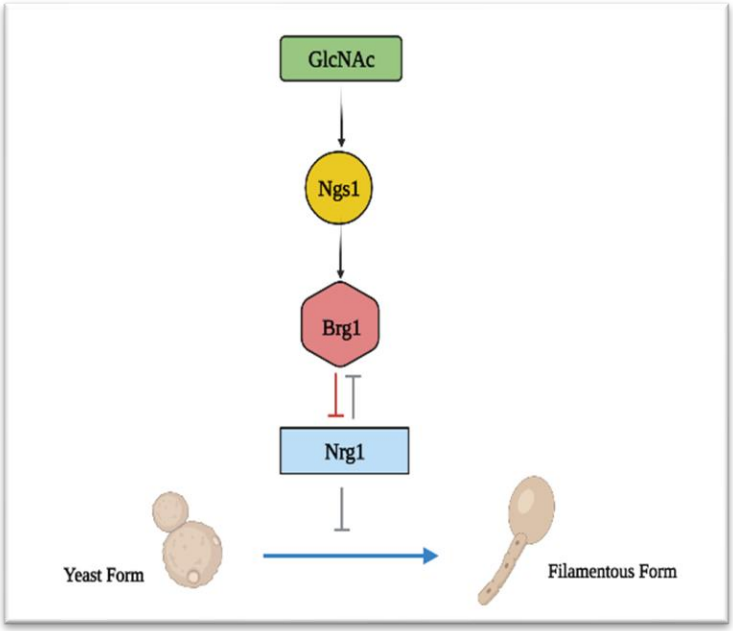
a slightly modified strategy that includes hyphal repressor transcription factor NRG1 and Biofilm regulator BRG1 to stimulate “Hyphal Specific Genes” (HSGs) [59, 64].

“GlcNAc transporter Ngt1”, a “sensor and transducer Ngs1”, and “Ndt80 transcription factor Rep1” are regulators for GlcNAc Signailing to produce GlcNAc-induced catabolic transcriptional responses [63]. Ngs1 operates as a key regulator of “GlcNAc signalling” in *C. albicans*, sensing GlcNAc and then triggering promoter acetylation of “GlcNAc catabolic genes” to activate gene expression. “Rep1” is an essential “Ndt80-family transcriptional factor” with a “DNA Binding Domain” [63], which likely has particular active sites on promoters of “N-Acetylglucosamine catabolic genes” to enlist “Ngs1” to couple gene promoters. Ngs1 and Rep1 are both upregulated and have been linked to “N-Acetylglucosamine catabolic gene promoters” in a “GlcNAc-independent” manner, but their stimulation is “GlcNAc dependent”. “Ngs1” has two domains. One N-terminal restored “3-Glycoside Hydrolase” (GH3) domain, that again is linked to the bacterial – “N-acetylglucosaminidase domain” [65], and exhibits distinct GlcNAc specificity [63], and the C-terminal “GCN5 linked N-acetyl transferase (GNAT) domain,” that attains an intrinsic “histone acetylase” activity [56]. These two operationally distinct domains participate in “GlcNAc-induced promoter activation,” chromatin acetylation, and subsequent transcription of several GlcNAc metabolic genes.

Most notably, Ngs1 regulates the expression of HSG by straightway inducing transcription of “BRG1” via promoter acetylation. Brg1 is a transcription factor of the “GATA family (Gat2)” that was initially identified as a “Biofilm Regulator (BRG1)” [66]. Ngs1 GNAT domain's histone acetyltransferase activity slackens the nucleosome binding at the “BRG1 promoter”, resulting in BRG1 upregulation. Brg1 inhibits “NRG1”, a universal repressor of filamentation [67], and inducts “Hda1, a histone deacetylase”, to the promoters of HSG to accomplish nucleosome re-positioning, concealing the Nrg1 “DNA binding site” [59, 68, 69]. GlcNAc induces and maintains hyphal induction and maintenance via Brg1-conciliated downregulation of “Nrg1” expression, which is triggered by GlcNAc discerning by “Ngs1”. During the signalling process, non-phosphorylated unbound GlcNAc straightway binds to the “GlcNAc sensor (Ngs1)” to advance “histone H3 acetylation” of the nucleosomes at the promoters of “GlcNAc catabolic genes” [63], Working representation depicting the induction of “GlcNAc catabolic genes” via “Ngs1-Rep1” mediated. The chromatin-modifying actions of “Ngs1-Rep1 complex” activator cause changes in chromatin structure like slackening of nucleosomes, which promotes the transcription of related genes. This chemical change neutralizes histone

tails positive charge, relaxing “histone-DNA interactions” and allowing various transcription factors to reach promoter chromatin for transcription.

(A)



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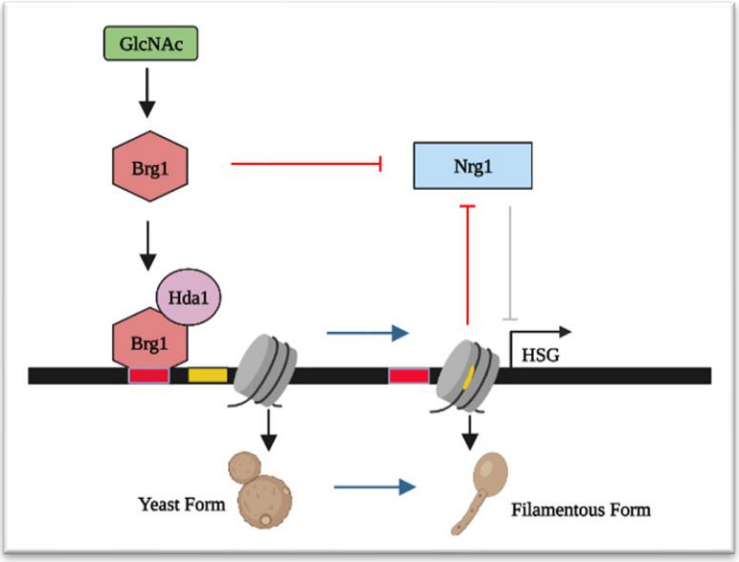


Figure 4: Two modes of regulation of GlcNAc-inducing filaments in *Candida albicans.* (A) Through the action of its histone acetylase, the GlcNAc sensor (Ngs1) that is activated upregulates BRG1 in response to GlcNAc binding or sensing. In order to enhance hyphal transition, the accumulating Brg1 stimulates the expression of the hyphal-specific gene (HSG) by down-regulating the NRG1 repressor. Under these circumstances, this process takes place in "log phase" cells at 37°C in the absence of fresh-medium injection. (B) BRG1 is activated under inoculation circumstances in the presence of GlcNAc, serum, and other inducers

resulting in hyphal maintenance. Histone deacetylase Hda1 and Brg1 interact to prevent Nrg1 from binding to regulatory sites, which in turn induces nucleosome repositioning at HSG promoters and downregulation of NRG1 expression. Grey lines are used to symbolize inactive relationships, whereas black and red lines are used to show active relationships.

1.10 Effect of Amphotericin B on Glucose and GlcNAc Induced cells:

The azoles, polyenes, and echinocandin groups of antifungal medications are the most successful in treating Candida infections. The polyene "amphotericin B (AmB)", discovered in the 1950s, is the antifungal drug often used in clinical practise to treat both superficial and systemic candidiasis [70, 71]. According to the main concept, which is now generally recognized, AmB exerts its action by attaching to ergosterol within the "fungal cell membrane", causing changes in the "selective membrane permeability" that ultimately result in "cell death" [72, 73, 74] According to Palacios et al., AmB's fungicidal action is not dependent on its capacity to generate holes at the plasma membrane [75]. Investigations have shown that the "AmB-induced cell death" in *C. albicans* was exacerbated by the development of oxidative stress via the formation of "reactive oxygen species (ROS)". The expression of genes related to cell stress, ergosterol production, and small-molecule transport was impacted by AmB [76, 77, 78].

A polyene antifungal medication called amphotericin B (AmB) is frequently used to cure systemic fungal infections [79]. The metabolic properties of "*C. albicans"* cells manifested to AmB were characterized in a metabolomic investigation utilizing "gas chromatography and mass spectrometry" (GC/MS). Thirty-one metabolites that were differently generated between the "AmB-treated" and control groups were found; 10 of these metabolites had upregulated levels, and 21 had downregulated levels. The major functions of these differently generated metabolites were in the production of polyamines, "tricarboxylic acid" (TCA) cycle, glycolysis, "oxidative stress", "glutathione metabolism", and "lipid synthesis". Subsequent research revealed that the polyamines spermidine, spermine and putrescine were crucial in determining how sensitive *C. albicans* cells were to AmB treatment, suggesting that combining AmB with inhibitors of the "polyamine production pathway" might be a useful antifungal tactic. This work offered fresh information on the mechanistic action of antifungal drugs by providing a systemic perspective of the metabolic pathway in "*C. albicans"* following subjection to AmB [80]. Further, we have revealed the effects of AmB on GlcNAc-induced cells also with respect to previous studies on wildtype strains.

2. Review of literature

2.1 *Candida albicans*:

With death rates advancing 40% despite treatment, "*Candida albicans"* has been the primary cause behind serious invasive infections in recent decades. With a rising number of "immunocompromised patients" [81] the widespread need for "organ transplantation" and the frequent utilization of antibiotics and immunosuppressants in "cancer chemotherapy" [82, 83], the clinical Candida septicaemia incidence rises year after year [13, 83-86]. *C. albicans* was detected in 70% to 90% of candidiasis caused by fungi [87]. *C. albicans* is also responsible for many candidiasis patients, with a mortality rate of up to 43.6% owing to candidemia [88].

Over the last few decades, we have seen a troubling trend in which traditional antimicrobials have been more ineffective at combating infectious illness. With the present scenario frequently referred to as a post-antibiotic era, there is a significant demand for the development of innovative therapies to battle "pathogenic microorganisms," additionally need to forecast and prevent the emergence of defiance to our present antimicrobial arsenal. This issue of antibiotic defiance is of particular importance in the conditions of "fungal pathogens" as there is lack of separate classes of antifungals for the treatment of protruding infections and the introduction and expansion of "multidrug-resistant fungal pathogens." Despite their catastrophic influence on human health, pathogenic fungi are an unappreciated contribution to human illness and death. *Candida, Cryptococcus,* and *Aspergillus* species are the most common etiological agents of systemic fungal infections, accounting for more than 90% of mycotic mortality [89, 90]. Candida species are the major prevalent cause of "invasive mycotic" illness in people who are highly immunocompromised, have had intrusive clinical operations, or have suffered catastrophic abrasion that necessitates prolonged treatment in critical care facilities. Even though fungi are detrimental to human well-being, few kinds of antifungal medicines are now available for curing these potentially fatal illnesses. The evolution of "novel antifungal drug" has been delayed, owing to the "eukaryotic structure of fungal cells," problems with chemical permeability across the membrane and cell wall of fungi, and a lack of interest in creating novel antifungals from the pharmaceutical sector [91-93]. The rising incidence of fungal isolates with "innate or acquired resistance" to one or more medication classes threatens our limited repertory of therapeutically useful antifungals.

2.2 Different morphological forms of *Candida albicans*:

One distinguishing feature of "*Candida albicans"* is that it may live in three phases like budding "yeast, pseudohyphae, and hyphae" [85]. The "mycelial form's" flexibility is a predictor of "drug resistance" and a key form throughout the infection stage [86]. The "hyphal form" is one of the main factors in the infection process because it promotes "tissue penetration" and "immune cell escape" [9, 94]. Hyphal morphogenesis is linked to virulence because genes controlling hyphal morphology and the genes that code for virulence factors are co-regulated. These genes code for adhesion [95, 96], hydrolase secretion [97], and candidalysin [39]. Significant technological improvements in recent years have aided research into the molecular biology of hyphal induction and proliferation. The research demonstrating the molecular process for hyphal induction throughout the infection phase has improved our understanding [64, 98, 99]. The amazing morphological flexibility that allows "*C. albicans"* to adapt to different host conditions may justify this commensal's pathogenic potential.

2.3 Different ways of Hyphal induction:

2.3.1 With Inoculation

To stimulate yeast-to-hypha transformation, saturated cells are diluted into new media at 37 °C. The transcription factor Nrg1 is critical for suppressing hyphal growth [36, 69; 100, 101]. Ectopic NRG1 expression suppresses hyphal filamentation in every in vitro growing condition as well as during invasive infection, resulting in reduced pathogenicity in a systemic infection model [102, 103]. With induction conditions of 37 °C and inoculation, disabling the transcriptional repression by "Nrg1" results in the "yeast-to-hypha" change, in which Nrg1 dissipates quickly by "transcriptional down-regulation of NRG1" and deterioration of Nrg1. The "cAMP-PKA pathway" is required for diminished "NRG1" expression through hyphal induction as "adenylyl cyclase (Cyr1)" or Tpk2 "catalytic subunit of PKA" is necessary [104]. The "transcriptional down-regulation of NRG1" following hyphal induction requires a temperature of 37 °C. Heat shock protein 90 (Hsp90) appears to sense increased temperature and restrict hyphal growth since pharmacological suppression of "Hsp90" by geldanamycin results in hyphal development.

The vigor of hyphal actuation and "Nrg1 down-regulation" are influenced by nutrients and environmental factors [64]. For instance, hyphal induction and Nrg1 elimination occur more

slowly in mannitol-containing media than in glucose-containing medium, which is consistent with glucose activating the cAMP-PKA pathway. Additionally, "N-Acetylglucosamine," CO_2, or the "bacterial peptidoglycan" present in serum are examples of cues that drive hyphal growth and are known to be integrated by Cyr1 [61, 105, 106]. The resilience of hyphal induction can be improved by these signals, even if they are not necessary for the "down-regulation of Nrg1" in the course of hyphal actuation caused by 37 °C and inoculation.

The inoculation process is another prerequisite for hyphal growth "in vitro," in addition to the 37 °C growth temperature or nutritional signals. "Farnesol, a quorum-sensing" chemical produced by "*C. albicans,"* is released from its inhibition in the used medium [107]. Farnesol primarily inhibits hyphal initiation by preventing "Nrg1 protein degradation". The Cup9 transcriptional repressor is destroyed after inoculation when cells are liberated from "farnesol inhibition," permitting the production of "Sok1" and successive stimulation of "Nrg1 protein degradation" [108]. As a result, two distinct regulations, one "cAMP-dependent transcriptional down-regulation of NRG1" and the other "degradation of Nrg1 protein," which is prompted by the liberation from farnesol suppression during inoculation. This way *C. albicans* achieve the temporary clearance of "Nrg1 protein" throughout "hyphal induction." Both routes are necessary for the quick removal of "Nrg1 protein". Neither one is enough for hyphal growth when inoculation settings are present.

2.3.2 Without inoculation

In "log-phase cells" at 37 °C, it has been discovered that exogenous stimuli like "N-Acetylglucosamine", neutral pH, or serum may effectually induce hyphal development [60, 63, 109, 110]. These findings showed that "*C. albicans*" may produce hyphae in various cell states. This flexibility may help *C. albicans* deal with varied host environments while infected [183]. "Downregulating Nrg1" is also important for hyphal formation in "log-phase cells", much like "hyphal induction" with inoculation [111]. The hyphal actuation and "Nrg1 down-regulation", however, occur considerably more gradually than the inoculation-induced response. This may be explained by the lack of apparent "Nrg1 degradation" in "log-phase cells" through hyphal growth when the impact of farnesol inhibition release is absent [111]. In the absence of dilution, the decline in Nrg1 protein is due to the "transcriptional down-regulation of NRG1". The NRG1 extent drops significantly for at least 4 hours during hyphal development in "log phase cells", as opposed to the temporary absence of Nrg1 during inoculation, when Nrg1 is regained after 1 hour [64]. As the temperature increases from 30 to 37 °C, a burst of cAMP is produced during hyphal formation. However, this burst is insufficient to permanently downregulate

NRG1 [106], which may be the reason why log cells require additional external stimuli for hyphal induction. Long-term effects on the actuation of "BRG1 expression," that represses "NRG1 transcripts" to promote filamentation, may be obtained from "N-Acetylglucosamine, serum, or neutral pH" [111]. The result that Nrg1 inhibition is important for morphological flexibility in *C. albicans* underlines the importance of eliminating it. "Nrg1 down-regulation" is required for hyphal development under settings irrelevant of inoculation.

2.4 Different elements for Hyphal induction:

Mucosal infections are frequently brought on by *Candida albicans*. Additionally, it can lead to fatal bloodstream infections that spread to internal organs in some immunocompromised people. The fungus may develop in "yeast, pseudohyphae and hyphal forms," making it polymorphic. The "hyphal form" can enter the circulation and destroy tissue by penetrating epithelia and endothelia. *C. albicans* develops hyphae in reaction to signals like "37 °C temperature," "O_2 and CO_2 tension," serum, and neutral pH because it is incredibly responsive to the many conditions that it experiences in the "human host." The presence of bacterial cells as well as other *C. albicans* cells, both of which are detected by quorum sensing chemicals, also controls the morphological flip. The "sesquiterpene farnesol," which is released into the environment and prevents hyphal growth [112], is used by "*C. albicans* cells" to feel the density of its circumambient population. Tyrosol, an aromatic alcohol, increases the growth of hyphals and germ tubes in biofilms and shortens the lag period of dormant yeast cells.

Multiple upstream pathways are activated by environmental signals to feed a console of transcription factors. The transcription factor boosted "filamentous growth protein 1 (Efg1)" is targeted by the cyclic AMP-dependent pathway, which is expected to have a significant role. "Adenylyl cyclase" integrates many signals in this route in both "Ras-dependent and independent" manner. "DNA-binding proteins" like "Nrg1" and Rox1p alike regulators of filamentous development target the promoters of genes unique to hyphae in order to exert negative control through the generic transcriptional corepressor Tup1 (Rfg1) [58].

2.5 Cell biology of Hyphae formation from Yeast form:

"Germ tube" emerges from the "mother cell," while "unbudded yeast cells" are prone to generate hyphae. A smear of septins, which later form a strap at the "germ tube's" bottom and a cap at elongating extremity, identify the beginning of evagination. The germ tube only displays strongly polarised development because it only elongates from its tip. The first "cell cycle" begins once the "germ tube" has developed and sizes as long as 15 to 20 µm [13, 113 -

116]. The cap of septins apparently forms a ring throughout the germ tube tip at the same time as the cell cycle begins [113, 115]. This ring is set in place as the tip lengthens. After the first mitosis, one descendant nucleus drifts backward into the “mother cell,” while the other moves to a location on the crestal side of the “septin ring” [113, 115]. The “mother cell's nucleus” then moves into the “germ tube.” Actin and a type II myosin 1 (Myo1), with myosin light chain 1 (Mlc1) as its regulatory side chain, are the components of the actomyosin ring. These are generated when the septin ring splits into two [117]. Among the two “septin rings,” one formed of chitin synthesized by “chitin synthase 1 (Chs1)” or Chs3 enzymes, the actomyosin ring compresses, directing the creation of the main septum [118]. Secondary septa are created by invaginating cell wall expansion after the original septum is formed. The two daughter compartments stay tightly linked to one another because the main “septum” is not hydrolysed after the development of the secondary septa, as it is in yeast cells. The distinctive hyphal form is a lengthy “tube-like structure” having aligned sides along its whole length since cytokinesis does not cause any constriction [12, 119].

The subapical compartment known as a “hypha” becomes extensively vacuolated and stays in G1 after cytokinesis [120]. The hyphal tip keeps growing and the crestal compartment stays in the “cell cycle,” which causes the cycle to recur with the sub crestal compartment vacuolating and staying in G1 each time. The vacuoles shrink and cytoplasm steadily accumulates in the subapical compartments at a rate that depends on the environment's level of nutrient richness. A threshold level of cytoplasm is eventually reached, allowing re-entry into the “cell cycle.” Thus, the “mother cell” may produce a “second germ tube,” while other sub crestal compartments produce branches where the septa are formed. Mature “*C. albicans”* hyphae are poorly branched because this happens at a rate far more moderate than the “cell cycle” happening in the crestal compartment [121].

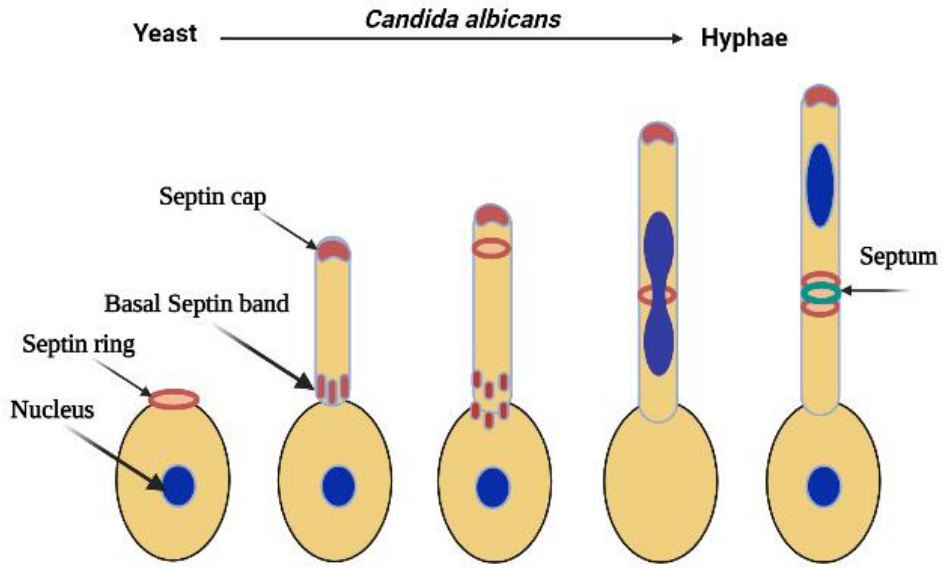


Figure 5: Cell biology of hyphal *C. albicans* cell. First "septin ring" then "septin band" and "septin cap" is formed. After that the nucleus gets divided and a septum is produced while switching from yeast to hyphae form [113].

2.6 GlcNAc Catabolism, Anabolism and Signailing process:

The peptidoglycan coating of "bacterial cell walls", the chitin shell of fungi, and "parasite cell walls" contain the amide-derived sugar N-Acetlyglucosamine (GlcNAc). Many microbes use GlcNAc as a source of nutrition, and it also has a crucial role in both bacterial and mammalian cells in signalling [54,122,123]. It controls several biological functions, such as morphological changes, pathogenicity, the body's reaction to oxidative stress, antifungals in *Candida albicans*, and cell death [61,62,124]. Given that GlcNAc is present in both "mammalian and microbial cells," it is expected that it would be plentiful in the human host and may play an important signalling molecule that controls the change for the Candida species betwixt commensalism and pathogenicity.

An evaluation of *C. albicans* proteomes in comparison. The protein Ngt1 (GlcNAc-specific) was discovered in yeast and filamentous cells localized to the plasma membrane. The first eukaryotic GlcNAc-specific transporter [125]. Ngt1 is a membrane transporter with 12 transmembrane domains that shares structural similarities with MFS membrane transporters. With *C. albicans* capabilities, GlcNAc sensing and signalling are probably linked. *C. albicans* to communicate with and avoid the immunological reaction of the host. "Ngt1, a transporter" that takes in GlcNAc, "Hxk1, a kinase" (EC 2.7.1.59) that turns GlcNAc into "GlcNAc-6-

phosphate", "Dac1, a deacetylase" (EC 3.5.1.33) that turns "GlcNAc-6-phosphate into glucosamine-6-phosphate", and "Nag1, a deaminase" (EC 3.5.99.6) that turns "glucosamine-6-phosphate" into glucose-6-phosphate are the enzymes required to catabolise [50, 51, 126]. The processes involved in the metabolism of "GlcNAc-6-phosphate", after it is generated, are poorly understood for animals in which GlcNAc usage appears to be rather widespread (apart from the GlcNAc catabolic route) [60]

Sensitivity against self-produced "GlcNAc-6-phosphate" is provided by the detection of exogenous, "non-phosphorylated GlcNAc". The internal de novo generated GlcNAc-6-P is not a part of the "GlcNAc signalling system"; instead, it is used in anabolic processes like chitin formation, N-glycosylation, and GPI-anchors. "GlcN-6-Phosphate synthase (Gfa1)" and "GlcN- 6-Phosphate acetyltransferase (Gna1)" work together sequentially to create GlcNAc-6-P from "fructose-6-Phosphate". (Glycolysis). GlcNAc-6-P is transformed further to "UDP-GlcNAc" by the successive actions of "UDP-GlcNAc pyrophosphorylase" (Uap1) and "phosphor acetylglucosamine mutase" (Agm1) [51, 56].

The GlcNAc-driven catabolic transcriptional responses are produced by GlcNAc signalling in *Candida albicans*, which is mediated by the "N-Acetylglucosamine transporter Ngt1", the "sensor and transducer Ngs1", and the "Ndt80 transcription factor Rep1" [63]. *C. albicans* have a main regulator of "GlcNAc signalling" that recognizes GlcNAc and then causes promoter "acetylation of N-Acetylglucosamine catabolic genes" to activate gene expression in "Ngs1". Rep1 is a crucial member of the "Ndt80 family of transcription factors". It features a DNA Binding Domain (DBD) that likely binds to particular places on the "promoters of catabolic genes" in order to attract Ngs1 to related gene promoters [63]. Thus, through Ngs1-modified transcriptional responses, GlcNAc activates a variety of cellular functions including catabolism, sugar scavenging, transport, hyphal transformation, pathogenicity, and numerous uncharacterized transcript regulators [56].

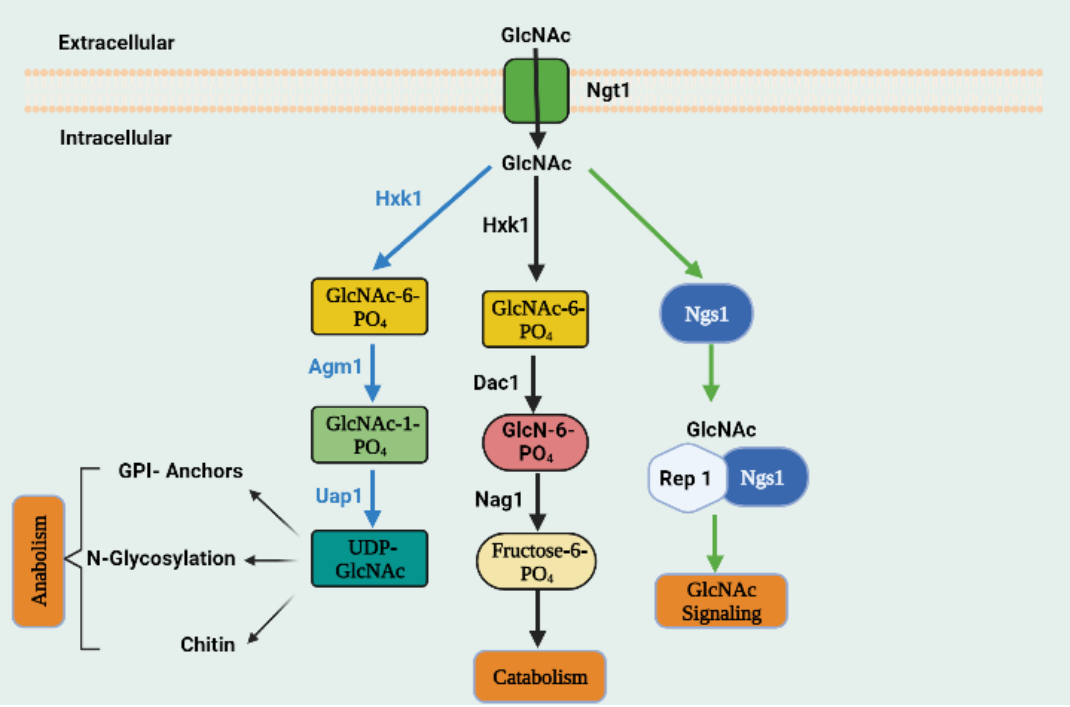


Figure 6: Different roles of N-Acetylglucosamine (GlcNAc) in *Candida albicans.* GlcNAc helps in Anabolism, Catabolism, and Signaling processes.

2.7 Antifungal activity and impact on *Candida albicans*:

Over the last several decades, we have seen a troubling trend in which traditional antimicrobials have become more ineffective in combating infectious illness. Given the scarcity of diverse groups of antifungals for the cure of invasive infections, as well as the introduction and dissemination of "multidrug-resistant fungal pathogens," the problem of antimicrobial resistance is of relevance in fungal pathogens. The frequency of major fungi-caused illnesses has escalated in recent decades due to an increase in immunocompromised persons, such as cancer patients, organ transplant recipients, HIV-infected individuals, and an aging population.

Healthy individuals frequently have *C. albicans* as a non-harmful commensal in the mouth or gastrointestinal system; but, in severely immunocompromised patients, Candida can spread in the bloodstream and "colonize internal organs," causing certainly fatal "systemic infections." Even with the harmful effects that fungi have on human health, there are currently just a few kinds of "antifungal drugs" that can be used to treat undoubtedly fatal diseases caused by fungi. Due to the "eukaryotic structure of fungal cells," problems in compound "permeability" through the "fungal cell wall and membrane," and apathy from the remedy sector, the

development of innovative antifungal medicines have often been delayed [91- 93]. The escalating incidence of fungus isolates with “inherent or acquired resistance” to one or more medication classes poses an additional danger to our small arsenal of therapeutically useful antifungals. We reviewed the antifungal drugs on the market, the defenses developed by Candida species against them, and any potential fresh treatment avenues that may be investigated to fight these diseases.

2.8 Primary Antifungal drug classes:

2.8.1 Polyenes

The earliest family of antifungal medications used to cure inherent fungal contaminations are polyenes, which are organic, amphipathic compounds. Amphotericin B, the most widely used polyene, has powerful action against a variety of clinically important “fungal species,” like several *Candida, Cryptococcus,* and *Aspergillus* species [127, 128]. According to conventional wisdom, the polyenes immediately bind to ergosterol and create “drug-lipid complexes” that interpose into the membranes of fungal cells, leading to the release of internal components and eventually cell death [128]. Recent structural and biophysical work, however, have cast doubt on this model, showing that amphotericin B (Amp B) forms an extra membranous mass that acts as a fungicidal "sterol sponge," removing ergosterol from fungal cell membranes. Despite its broad-spectrum antifungal action, amphotericin B is not often used in medicine [184]. This is mainly because it has a low oral bioavailability and harmful effects on the host that are dose-dependent and derive from the similarity among ergosterol and cholesterol [128, 129]. Fortunately, *C. albicans* seldom develop amphotericin B resistance, perhaps as a result of the high fitness costs involved in the development of polyene resistance [130, 131].

2.8.2 Azoles

The most often used medication class in clinics has been the azoles. By impeding the production of ergosterol, a crucial component of the “fungal cell membrane,” these heterocyclic synthetic chemicals operate as antifungal agents by interfering with “membrane stability”, permeability, and the activity of “membrane-bound enzymes” [127, 132]. The buildup of hazardous sterol intermediates, like “14-α-methy-3,6-diol”, is another effect of azole exposure [127, 132]. The azoles specifically bind to the heme group in the active site of the “cytochrome P450” enzyme such as “lanosterol 14-α-demethylase”, which is encoded by

“ERG11” in *Candida* species [133]. Many azole antifungals, in contrast to others, have great “oral bioavailability” and are offered in both “oral and intravenous” forms [134]. A notable disadvantage of azoles is that they impede mammalian “cytochrome P450 enzymes,” which oversee “drug metabolism”. This increases the likelihood of drug interactions [91]. New azoles “VT-1129, VT-1161 and VT-1598” with improved selectivity for fungal enzymes are now being developed to get around this restriction [135 - 138]. A further drawback is that azoles do not really kill *Candida* and *Cryptococcus*; rather, they just stop their growth, which exerts significant guiding selection pressure and encourages the establishment of “antifungal drug resistance.” Different species of *Candida* have varying degrees of azole susceptibility. *C. albicans* is often responsive to fluconazole, the most widely used azole.

2.8.3 Echinocandins

Echinocandins are natural derivatives made up of a “cyclic hexapeptide core” and “N-linked fatty-acyl side chain.” Only new antifungal medication class to reach clinical practice in decades. “Echinocandins” operate as antifungals by destroying the cell wall, which is totally lacking in mammalian cells. The catalytic component of “1,3-β-D-glucan synthase”, which is enciphered by “FKS1” in *C. albicans*, binds to them non-competitively. A breakdown in the integrity of the cell wall and disparity in osmotic pressure caused by a halt in the manufacture of “1,3-β-D-glucan” result in a fungicidal impact [139]. Given their powerful action against *Candida* species, echinocandins are currently advised as a frontline therapy for “invasive candidiasis” [140, 141]. Echinocandins have several benefits, including a superior safety profile, increased fungal selectivity, and a decreased risk of “drug-drug interactions” [142]. Echinocandins, like polyenes, have poor “oral absorption,” hence their usage in clinical settings is constrained to intravenous delivery [143]. Ibrexafungerp, a “1,3-β-D-glucan synthase inhibitor” that is structurally unique and is presently being tested in “phase II and phase III clinical studies,” is now available in oral and intravenous forms [144]. Due to echinocandins' extensive therapeutic usage, resistance has unavoidably developed in recent years. Fortunately, echinocandin susceptibility is still high for other clinically significant *Candida* species, such as *C. albicans* [145].

2.9 Effect of Amphotericin B on *Candida albicans*:

Invasive, potentially fatal fungal infections can vary from superficial, to systemic diseases. Invasive fungal infections can result from fungal cells invading the epithelial side of immunocompromised hosts. There is a substantial morbidity and death rate linked to these

invasive fungal diseases. About 50–60% of cases of candidiasis are caused by *C. albicans*, the main human opportunistic fungal infection, particularly in immunocompromised people. Infections brought on by *C. albicans* are frequently treated with azole medications [146, 147]. In patients who were nearing the end of their lives, amphotericin B was still utilized to treat fungus infections [148, 149]. By attaching to ergosterol in the membrane of the fungus and causing membrane permeabilization via channel creation, AmB kills yeast [150, 151]. This fungicidal medication can leave patients with severe nephrotoxicity, chills, and convulsions. Despite its toxicity, AmB is still employed in clinics due to its effectiveness. Better medications need to be made that are less harmful [152, 153]. A key present worry is the emergence of drug resistance amidst the main human fungal infections, such as *C. albicans*, and the development of "drug-tolerant biofilms" on both biotic and abiotic surfaces. For instance, most antifungal drugs are ineffective against *C. albicans* biofilms [154].

So, it comes to reason that understanding how AmB destroys fungus cells will also help in the crucial quest for novel forms of resistance-refractory antimicrobial medicines. However, while being the last resort for the cure of systemic fungal infections that pose a serious threat to life, AmB has significant dose-limiting adverse effects [155, 156]. A more thorough molecular comprehension of this drug's method of action would greatly aid efforts to raise its therapeutic index. According to the main concept, which is now generally recognized, AmB employs its action by attaching to ergosterol present in the "fungal cell membrane," causing changes in the "selective membrane permeability" that ultimately result in "cell death" [72, 73, 74]. Recent structural and biophysical investigations, however, have cast doubt on this hypothesis, showing that amphotericin B forms extramembranous aggregates that function as a fungicidal "sterol sponge," removing ergosterol from fungal cell membranes [184]. Studies disclosed that the "AmB-induced cell damage in *C. albicans*" was exacerbated by the formation of oxidative stress by delivering "reactive oxygen species (ROS)." The antifungal mechanisms of AmB have been effectively clarified using microarrays and proteomics [76, 77, 78]. Experiments in gene expression profiling manifested that AmB had an impact on the "expression of genes" related to ergosterol production, cell stress, and small-molecule transport [79]. 43 AmB-responsive proteins were discovered by proteomic research, including those linked to oxidative stress and osmotic tolerance [157]. AmB's antifungal processes, however, are still not completely understood. This research produced an elucidation of the metabolic fingerprinting and metabolite alterations that point to the processes behind AmB's antifungal activity and *C. albicans'* stress response.

Finally, the "*C. albicans* metabolomic" investigation described here showed the modifications in metabolite synthesis in response to AmB. The creation of polyamines, the "TCA cycle", "oxidative stress", "lipid synthesis", "glutathione metabolism", and "glycolysis" are only a few examples of the specific subsets of metabolites that were noticed. AmB inhibits the formation of intracellular glutathione and lipid in *C. albicans* cells, which might lead to cell death. In order to protect themselves from the oxidative damage that the drug-induced stress causes, the cells increase trehalose production by lowering energy consumption. The formation of polyamines is another stress reaction, and it is this study's most significant discovery. Higher amounts of polyamines assist the cells to resist the effects of the antifungal medication by scavenging intracellular ROS more effectively [80]. AmB's ability to kill more organisms might be improved by interfering with the manufacture of polyamines. These findings provided fresh insight into the modes of action of AmB and showed the potential application of metabolomics to the study of antifungal drugs.

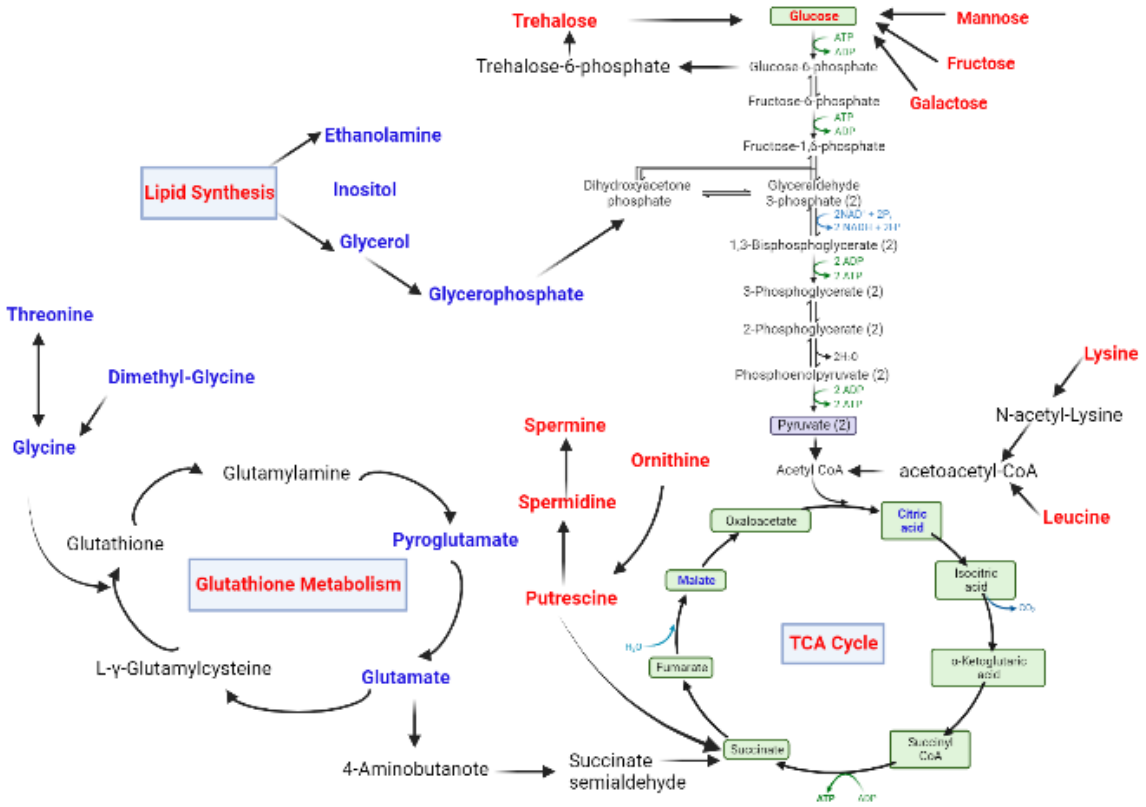


Figure 7: An illustration of how the metabolite changes among the AmB-treated and control non-treated wild-type Candida cells differ. The key metabolic processes affected by differential production include the synthesis of polyamines, the TCA cycle, glycolysis, glutathione metabolism, oxidative stress, and lipid synthesis. AmB therapy results in a drop in intracellular glutathione and lipid levels, which lead to "cell death." In order to protect themselves from the oxidative damage caused by AmB, the cells increase trehalose production while decreasing energy consumption as a stress response. The increase of polyamines, such as putrescine, spermine, and spermidine are another significant stress response. Higher amounts of polyamines assist the cells to resist the effects of the antifungal medication by scavenging intracellular ROS more effectively. The red-hued metabolites correspond to those that were elevated in the AmB-treated group. The blue-hued metabolites correspond to those that were downregulated in the AmB-treated cells.

3.1 Objectives:

- Creating mutants of HXK1 of *C. albicans* and analysing.
- Growing different mutants as well as wildtype strains of *C. albicans* in Yeast Extract Peptone Dextrose (YEPD) and Yeast Nitrate Base (YNB)-GlcNAc/YNB-Glucose medium.
- Determining various compounds (Metabolites) induced by GlcNAc signalling through metabolome analysis and anticipating their role in cellular metabolism.
- Checking different RNA profiles of the wildtype and the inducible genes caused by GlcNAc signaling.

3.2 Scope of the study:

When the recipient is vulnerable, the "human commensal yeast *Candida albicans*" transforms into infectious and causes heterogeneity of mucosal and deep tissue abnormalities. To live in the host niche environment and spread virulence, *Candida* demonstrates extraordinary metabolic plasticity and dynamic morphogenetic transition. In addition to serving as an excellent source of carbon and nitrogen, the "amino sugar N-acetylglucosamine (Glc-NAc)" present at the host infection area also triggers cellular signaling in this disease [185]. GlcNAc has a variety of functions in *C. albicans*, including scavenging, import, "metabolism," "morphogenetic transition" (switching from "yeast to hyphae" and from "white to opaque"), pathogenicity, and "GlcNAc-induced cell death" (GICD) [186].

How does commensal colonization begin, and how can *Candida albicans* survive for a long duration of time even with "host immunity" and "bacterial competition"? [187] What factors influence the switch from "commensalism to pathogenicity" in susceptible hosts? How does *Candida albicans* thrive in the vast range of environments it experiences as a "commensal and as a pathogen"? The questions needed to be answered. Most of the answers are elucidated but there are some ambiguities, which need to be addressed.

We hereby want to unfold the morphological, metabolic, and pathogenicity induced due to the induction of GlcNAc in *Candida albicans.* Additionally, previous research has shown that

blocking the GlcNAc utilization route reduces the pathogenicity of mice model candidiasis. It has been demonstrated that GlcNAc catabolism is crucial for growth and developing pathogenicity inside the host [188]. Even so, it is still unclear what role some metabolic enzymes play in the production of metabolic intermediates. Up until a few recent studies investigated the potential function of new metabolic enzymes implicated in GlcNAc metabolism. Moreover, we are investigating the effect of the "Antifungal agent Amphotericin B" on Glucose and GlcNAc-induced *Candida* cells.

4. Materials and Method

4.1 Media and culture condition:

Candida albicans (SC5314) cells were gifted from "Dr. Swagata Ghosh, Assistant Professor at the Department of Molecular Biology and Biotechnology, Kalyani University." *C. albicans* (SC5314) frozen stock in 15% glycerol was stored at -80°C for future use. Enough culture was streaked on YEPD agar "HiMedia-G038-500G" plates "(2% dextrose, 1% yeast extract, 2% Bacto peptone, and 2% Bacto Agar)" and kept at 28°C in a BOD incubator for 2 days to culture the fungal cells and start our laboratory work. Every week we cultured fungal cells on new YEPD Agar petri-plates to revive them and continue our work. Often, we checked the cells in Microscope to see whether the cells were pure or contaminated. Overnight cultures were cultivated in liquid YPD media "HiMedia-M1363-500G" "(2% dextrose, 1% yeast extract, and 2% Bacto peptone)" in 15 ml culture tubes rotating at "75 rpm at 30 °C" [158]. For an alternative carbon source, we also added 0.45 µm Syringe filtered 5miliMolar (mM) of GlcNAc and Glucose solution [159, 185]. The cells were procured in Yeast Nitrate Base with amino acids "HiMedia-M139-100G" and these carbon sources.

4.2 Preparation of HXK1 Mutant:

Using the procedure outlined [51], the HXK1 deletion mutant was created in the *C. albicans* strain SC5314. URA3 selectable marker gene (from plasmid pUC 19 CUB6) was amplified using specific PCR primers (HXK1-DEL-F and HXK1-DEL-R). PCR was performed using a set of primers that flanked the integration sites and primers that annealed into the integrated sequences to confirm that the deletion cassettes had been integrated at the correct locations. A plasmid containing one wild-type (WT) copy of *Candida albicans* HXK1 (CaHXK1) and the URA3 selectable marker gene was inserted into the *C. albicans* genome to create the supplemented strains (primer CHECK-URA3 was used for validation). The HXK1 mutant strain was gifted from "Dr. Swagata Ghosh, Assistant Professor at the Department of Molecular Biology and Biotechnology, Kalyani University" to do the proceeding works mentioned later.

4.3 Field Emission- Scanning Electron Microscope sample preparation:

At first, the "Glucose and GlcNAc-induced cells" were grown in YNB media at 30°C for 3 hours. *Candida albicans* suspension and 8000 rpm centrifugation are the typical preparation techniques for its scanning electron microscopy images. The cells were fixed in "2.5%

glutaraldehyde" for two hours in "0.1 M-$NaPO_4$ buffer," post-fixed in buffered 1% OsO_4 for two hours. After that washed with "phosphate buffer PBS" three times. For drying the material, increasing concentrations of ethanol were added: 30%, 50%, 70%, 85%, 95%, each once for 10 minutes, and 100% ethanol twice for 20 minutes [160]. "Critical point drying," "gold sputter coating", and FE-SEM examination of the specimens were the last procedures. [183].

When placing an intact sample into a scanning electron microscope example cell, we took care to ensure that the sample chamber's vacuum satisfies the necessary standards. Acceleration potential was then chosen; Adjusted the operating distance was in accordance with the relevant sample selection moveable aperture; adapted the appropriate multiplier; the focus length, image's overall brightness, and contrast were adjusted; photos were taken. In order to produce a clearer image when observing, it is important to notice any temporal adjustments to accelerating potential, object lens activity aperture, and operating distance.

4.4 "Minimum inhibitory concentration" (MIC) of *Candida albicans*:

Using the conventional broth microdilution method based on the proposition of the "Clinical Laboratory Standards Institute," it was investigated how Amphotericin B affected the proliferation of planktonic cells of *Candida albicans* [181]. Amphotericin B was prepared in Yeast Nitrate Base (YNB) medium in "untreated 96-well polystyrene plates" at various doses extending from 0.1 to 100 µg ml-1. Control wells were those lacking both Amp B and DMSO. To attain 1×10^3 cells ml-1, 100 µl of inoculum were varied with 100 µl of YNB medium in each well. 48 hours were spent incubating the plates at 30 °C. Using a "microplate ELISA reader (Labtronics model LT-1260)", the "absorbance at 620 nm wavelength" was measured to analyze the growth. The "minimum inhibitory concentration (MIC)" for *C. albicans* growth was elucidated as the lowest concentration of Amp B that resulted in a 50% decrease in absorbance in contrast to the control [182-183].

4.5 "High-Performance Liquid Chromatography (HPLC)":

C. albicans grown on YEPD plates and 1% inoculum is grown in YEPD media overnight for induction. Again 1% culture is grown in YNB-Glucose media for 12 hours. After examining the OD (nearly 1) it was centrifuged at 25℃, 6000 rpm for 15 mins and the pellet was cleansed with autoclaved water. At last, it is cultured in "YNB-Glucose" and "YNB-GlcNAc" for 3 hours at 30℃ and centrifuged at 4℃, 8000 rpm for 10 mins. Pellets were poured in liquid nitrogen, and the brittle samples were ground with a mortar pestle, homogenized in "methanol:

water” in a 3:1 ratio, vortexed with glass beads for a minute and in accordance with ice treatment for a min. This process was done thrice to lyse the cell membrane of *C. albicans*. Then cells were incubated for 1 hour and centrifuged to get metabolites. HPLC “HPLC-150722-70_160722_PDA_SS.lcm” with a Reverse phase column and PDA detector [183] was used to look for changes in proteins and metabolites in these samples.

4.6 “Gas Chromatography-Mass Spectroscopy (GC-MS)”:

“Gas chromatography-mass spectrometry (GC-MS)” is an inquisitive method that integrate the benefits of “gas chromatography” and “mass spectrometry” to detect distinct substances in a “test sample. It, like “liquid chromatography-mass spectrometry”, allows for the investigation and identification of even minute concentrations of a chemical. The "gold standard" for forensic substance detection is GC-MS, which is used to execute a “100% specific test” that emphatically identifies the existence of a particular molecule.

C. albicans was cultivated overnight in YEPD medium from YEPD plates with 1% inoculum for induction. From these 1% culture is cultivated for 12 hours on a “YNB-Glucose” medium. The pellet was cleaned with autoclaved distill water after “centrifuging at 25°C for 15 minutes at 6000 rpm” after examining the OD at 600nm wavelength. Later “centrifuged at 4°C, 8000 rpm for 10 minutes” after being cultured in “YNB-Glucose” and “YNB-GlcNAc” for 3 hours. After being submerged in liquid nitrogen, the pellets were lysed with a “mortar pestle,” homogenized in a 3:1 mixture of methanol: water, and vortexed with glass beads for one minute. This was followed by ice treatment for a minute. To lyse the cell membrane, this technique was carried out three times. The material was then dried in a “vacuum hot oven at 40°C for 3 hours” before being given a sample volume of 1µl for GC-MS ([GCMS-TQ8050 NX], D:\GC-Ms\Method\Carbohydrate-08062022.qgm) at CENTRAL INSTRUMENTATION LABORATORY [183] analysis using a hot needle method. A thermal gas chromatography-mass spectrometer fitted with a DB-5 capillary column (30 m length, 0.25 mm i.d., film thickness 0.25 µm) was used for the GC-MS analysis. The components were identified by comparing their mass spectra to the NIST mass spectral database and by comparing their retention indices to those reported in the literature or to those of real compounds. The result we got has been analysed using a “One-way ANOVA” and “Tukey's post hoc”.

4.7 "Liquid Chromatography and Mass Spectroscopy (LC-MS)" sample preparation:

The analytical chemistry method known as "Liquid Chromatography and Mass Spectroscopy (LC-MS)" unites mass spectrometry's mass examination ability with liquid chromatography's conventional separation capabilities (MS). Integrated "chromatography-MS systems" are frequently employed in chemical analysis due to the synergistic improvement of the unique characteristics of each technique. "Liquid chromatography" isolates mix that include various components, whereas "mass spectrometry" remit spectrum data that help in identifying (or confirming the putative identity of) each segregated component. "MS" is not only intuitive but also detectable. Which is the need for extensive chromatographic segregation. Due to its thorough coverage of diversity in compounds, LC-MS is also worthy of metabolomics. Analyzing biochemical, inorganic, and organic substances frequently present in composite samples of environmental and biological materials may be done using this tandem approach.

With 1% inoculum for induction, *C. albicans* is grown overnight in YEPD media from YEPD plates. On YNB-Glucose medium, 1% culture is once again grown for 12 hours. After OD testing, the pellet was centrifuged at 25°C for 15 minutes at 6000 rpm before being washed with autoclaved water. After being cultivated in "YNB-Glucose" and "YNB-GlcNAc" for 3 hours, it is then centrifuged for 10 minutes at 4°C and 8000 rpm. Liquid nitrogen was used to freeze the pellets, which were then homogenized in a 3:1 combination of methanol and water and vortexed with glass beads for one minute. The icing was applied for a minute after that. This procedure was three times repeated to lyse the cell membrane of *C. albicans*. Then the samples were sent for LC-MS analysis. The result we got has been analysed using a "One-way ANOVA" and "Tukey's post hoc".

4.8 RNA Extraction using the TRIzol method:

The Invitrogen TRIzol™ solution is a ready-to-use solution that can extricate high-quality total RNA from yeast cell samples within an hour, in addition to DNA and proteins. The TRIzol™ Reagent is a monophasic mix of phenol, guanidine isothiocyanate, and other unique components that facilitates the isolation of several RNA species with various molecular sizes. The TRIzol™ Reagent successfully inhibits "RNase activity" while breaking cells and dispersing cell components through sample homogenization, sustaining the integrity of the RNA. The single-step RNA extraction technique created by Chomcynski and Sacchi [161] is improved using TRIzol™ Reagent, which enables the handling of numerous samples

simultaneously. According to this process at first, the cells are lysed and separated into phases. After that to isolate the RNA, it is precipitated, washed, and solubilized. At last, the RNA yield was determined by using Agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific).

4.9 "Real time - Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)":

"Reverse transcription polymerase chain reaction (RT-PCR)" is a method used in laboratories that uses polymerase chain reaction (PCR) and complementary DNA, or cDNA, as the intended DNA in this case, to combine RNA to DNA amplification. It is usually used to determine how much of a specific RNA there is. A technique for doing this is real-time PCR, additionally referred as quantitative PCR, which uses fluorescence to track the amplification reaction. In research and clinical contexts, "combined RT-PCR and qPCR" are often employed for gene expression investigation and quantification of targeted RNA [162].

"The Real-Time One-Step RT-PCR Master Mix" was used to carry out the "real-time RT-PCR" test "Applied Biosystems." Every "25- µl reaction mixture contains" (i) 12.5 µl of the "2X Master Mix", (ii) 0.625 µl of the "40X MultiScribe" and "RNase Inhibitor" mix, (iii) 0.25 µl of the 10 µM probe, (iv) 0.25 µl of the 50 µM forward and reverse primers, (v) 6.125 µl of "nuclease-free water", and (vi) 5 µl of "nucleic acid extract". "96-well plates" and an "iCycler iQ Real-Time Detection System" were used for the amplification (Bio-Rad, Hercules, CA). "Thermocycling parameters were 45 cycles of 15 seconds at 95 °C and one minute at 60 °C, 30 minutes at 48°C for reverse transcription, and 10 minutes at 95°C for actuation of the AmpliTaq Gold DNA polymerase". Apart from the primer concentrations being employed, which were 30 µM each, the assay reaction was accomplished exactly as previously reported. In order to determine the "threshold cycle" (CT) value for all sample, fluorescence measurements were made, and the moment at which the fluorescence surpassed a "threshold limit" stow at the "mean + 10 standard deviations" (SD) over the baseline was identified. If two or more genetic targets produced positive findings (CT 45 cycles), clinical samples were deemed positive, and all positive and negative control responses produced the anticipated results. The result we got has been analysed using a "One-way ANOVA" and "Tukey's post hoc".

5. Result and Discussion

5.1 Growth of *C. albicans* cells using N-Acetylglucosamine (GlcNAc) as a solitary carbon source:

We observed *C. albicans* cells procured on "YNB-GlcNAc" plates with "YNB-Glucose" as a control by ensuing the same procedure as in Tao, L., et al., 2017 [163]. In order to completely comprehend the importance of GlcNAc as a carbon origin to support the growth and induce morphogenetic transition in "*C. albicans* (strain, SC5314)". We discovered that the strain can use "GlcNAc" as the sole carbon source, and the cells are elongated, flat and form a filamentous colony, as opposed to the spherical, smooth colonies produced by glucose-induced cells, as shown in Figure. For all effectors, the *C. albicans* strain testing procedures were the same. Cells were cultured in YEPD media. After reaching the "exponential phase," cultures were diluted to an "optical density" of 0.1 at 600 nm (OD600). On YNB plates containing 5mM of Glucose and GlcNAc, 4µl of "undiluted cell culture" and 1/5 successive dilutions of individual "cell culture" were spotted [185].

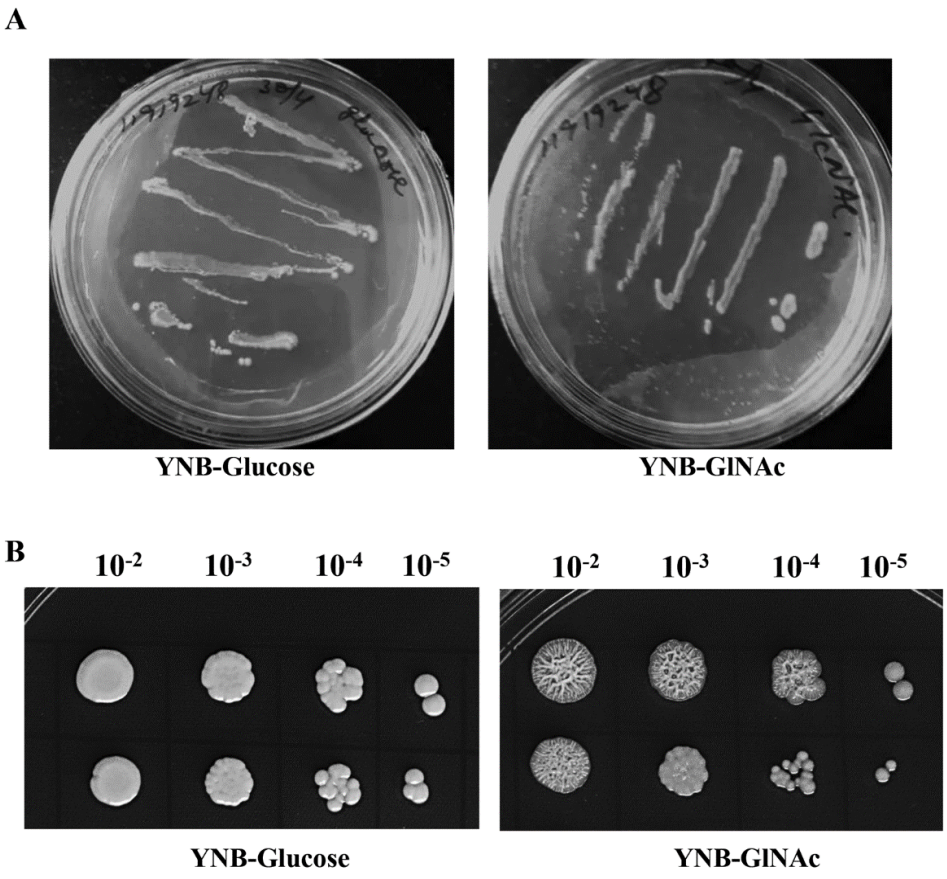


Figure 8: *Candida albicans* (SC5314) procured on "YNB-glucose" and "YNB-GlcNAc" media. A) Candida strain growth pattern after three days of growth at 30°C on "YNB-glucose and "YNB-GlcNAc" plates. B) For two days, a spot represents the development of the Candida strain cultured identically to panel A with different concentrations. Cells grown in GlcNAc are elongated, flat and form a smear colony, but cells produced in glucose are round and distinct in the colony [183].

5.2 Morphogenetic changes due to GlcNAc-induced *C. albicans* cells:

C. albicans can produce hyphae just by changes in the carbon source. As shown in Figure 9, while growing in a solution containing glucose encourages the development of yeast, using GlcNAc instead encourages the growth of mycelium. The "yeast cells of *C. albicans*" are comparatively elongated, and a clear separation from mycelial cells cannot be established until the germ tubes have become rather lengthy—a feature gained after a few hours of growth in the GlcNAc-containing medium. Following some time of incubation, the count of "mycelial cells" in the "YNB-GlcNAc" medium started to decrease, as was previously stated in [164]. Rather than the death of mycelial cells or the transformation of mycelial into yeast, this

decrease was brought on by the brisk reproduction of yeasts that had originally not responded to the morphogenetic stimuli. This finding raises concerns whenever the effect of external variables on dimorphism is examined. The combination of starvation and the development on GlcNAc produced the greatest results. After germination, 90% of the cell population developed into hyphal, and this proportion remained for 24 hours. After an additional 48 hours of incubation, extremely lengthy, branching strands appeared, but the quantity of yeast cells remained constant. This result proposes that the surviving yeast cells are dead as they are unable to bud. The microscopic images of the HXK1 mutant were also observed where we found somewhat boat-shaped and different from the wildtype as shown in Figure 10.

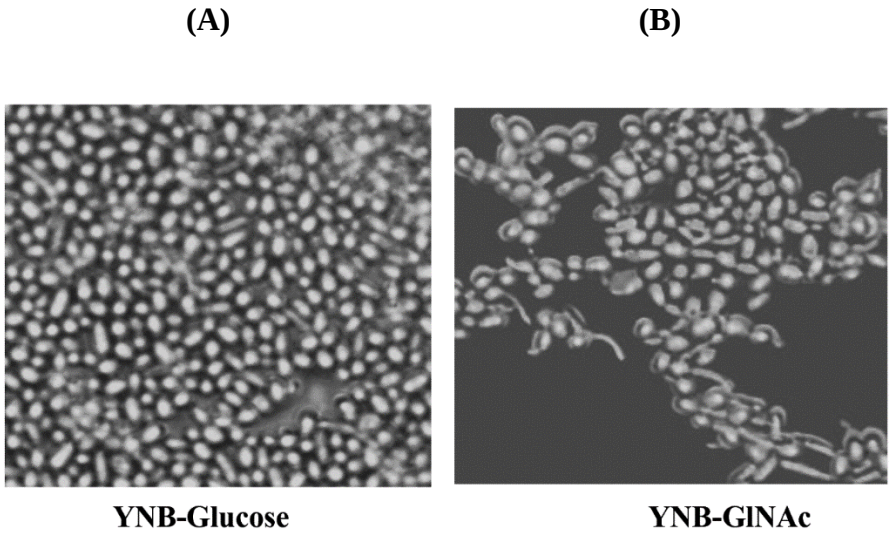


Figure 9: Microscopic image (40X) of *Candida albicans* (SC5314) procured on (A)"YNB-glucose" and (B)"YNB-GlcNAc" media. Cells grown in GlcNAc medium develop hyphae and are elongated in shape, whereas cells grown in glucose media are circular in shape [183].

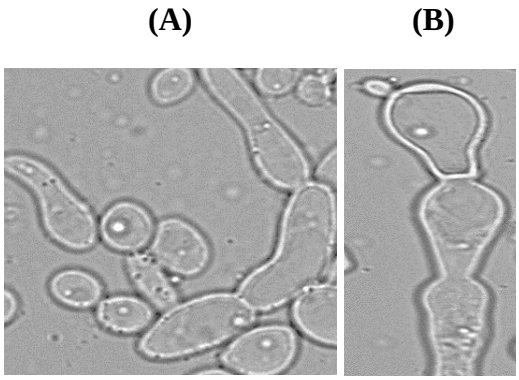


Figure 10: Microscopic image (100X) of ΔHXK1 strain of *Candida albicans*. We can clearly comprehend that this strain shows a somewhat boat-shaped morphology and is different from SC5314(A and B).

5.3 Field emission - Scanning Electron Microscope study:

Using a “light microscope,” the GlcNAc-treated cells' molecular differences from the glucose-grown control cells were visible (Figure). The image of *C. albicans* cells is taken using the unconventional technology familiar as “field emission scanning electron microscopy (FE-SEM).” Because gas molecules apt to derange the electron beam and the released “secondary and backscattered electrons” required for imaging, FE-SEM is usually carried out in a high vacuum. We intended to conduct an ultrastructural study of both “GlcNAc and Glucose-grown cells” before moving forward with differential metabolomics analysis. Following the steps outlined in the materials and methods part, we used a “Field Emission Scanning Electron Microscope (FE-SEM, Joel)” for this reason. The GlcNAc-treated cells displayed hyphal growth in comparison to the Glucose control cells. We can distinguish between them, and the study states that the typical “Glucose-induced cells” are “1.23 μm × 0.67 μm” in size, whereas the “GlcNAc-induced cells” are “9.60 μm × 3.80 μm” in size (Figure 11) [183].

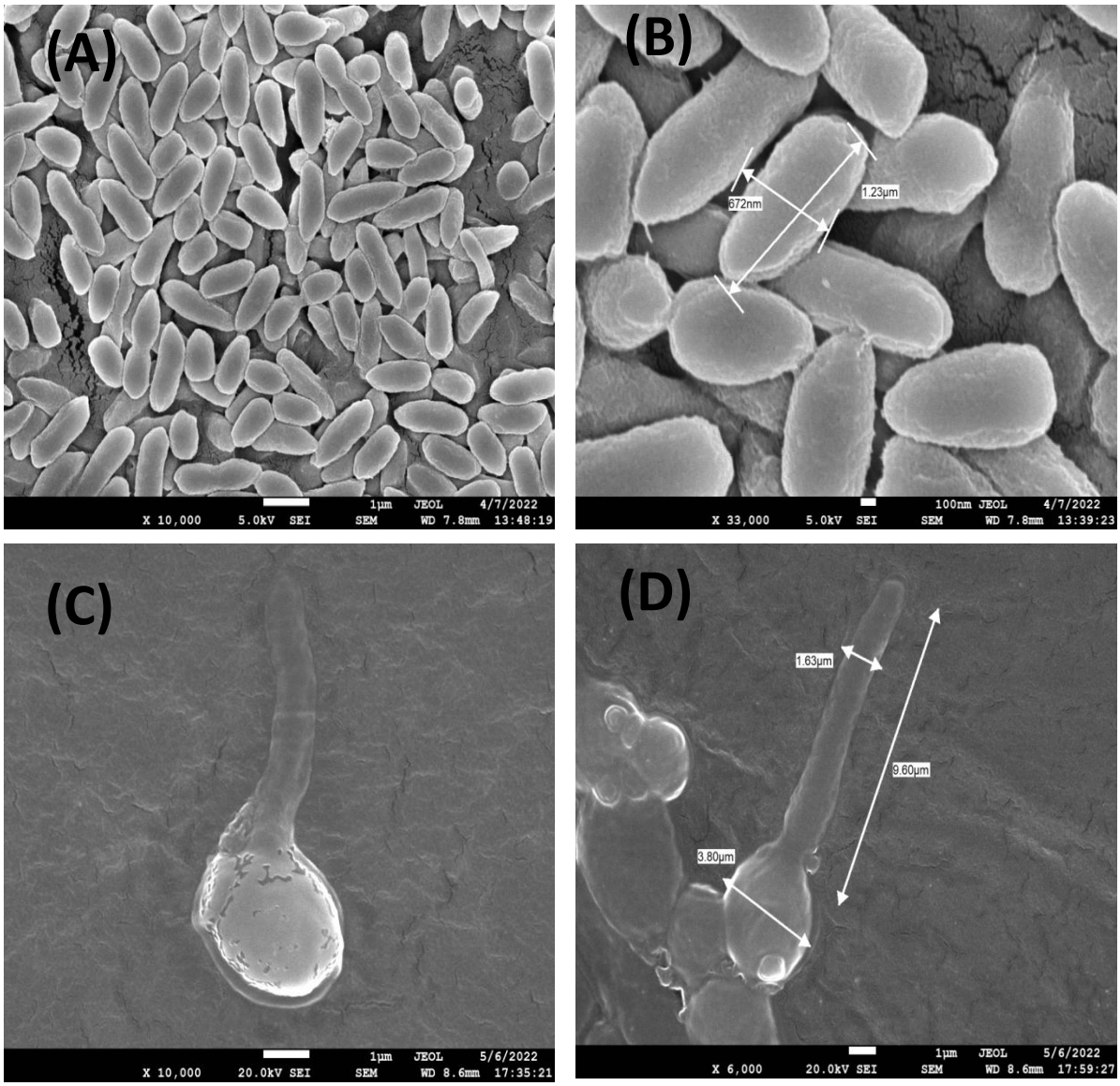


Figure 11: "FE-SEM" images of "glucose (Upper panel- A and B) and GlcNAc-induced (lower panel- C and D)" cells. Normal "glucose-induced cells" were "1.23 µm × 0.67 µm" in size, whereas "GlcNAc-induced cells" were "9.60 µm × 3.80 µm" in size. *C. albicans* yeast and hyphal forms may be readily distinguished. [183].

5.4 MIC analysis of "*C. albicans*" against Amp B:

The MIC of Amp B was 6 µg/ml for the suppression of *C. albicans* (SC5314) planktonic growth induced in glucose. Whereas the MIC for *C. albicans* (SC5314) induced in GlcNAc was 3 µg/ml. Different concentrations of Amp B were made in DMSO. As a control, we took fresh culture of YNB-Glucose and YNB-GlcNAc induced cells. The values of the MIC test are properly elucidated in the Table. At lower concentrations of Amp B GlcNAc induced cells are more affected. As in the budding stage or while hyphae formation if the cells are treated with Amp B then the cell wall is crusted with Amp B, Ergosterol-Amp complexes are produced in the cell membrane, and later on Amp enter the vacuoles and disrupt the cell.

Table 1: Absorbance at 620nm of various concentrations of AmpB used against *Candida albicans*.

Carbon	AmB	100 µg/ml	50 µg/ml	25 µg/ml	12 µg/ml	6 µg/ml	3 µg/ml	1.5 µg/ml	0.7 µg/ml	0.3 µg/ml	0.1 µg/ml	YNB+ DMSO (WC)*	YNB (WC)*
Glucose		0.4430 ± 0.203	0.3765 ± 0.175	0.7372 ± 0.564	0.7737 ± 0.598	**0.6877 ± 0.237**	0.859 ± 0.454	0.9010 ± 0.264	1.2517 ± 0.061	0.7246 ± 0.295	0.5367 ± 0.108	0.0264 ± 0.042	0.0330 ± 0.039
GlcNAc		0.0646 ± 0.007	0.1367 ± 0.008	0.1952 ± 0.116	0.2663 ± 0.125	0.7277 ± 0.060	**0.4962 ± 0.111**	0.4242 ± 0.022	0.4723 ± 0.012	0.9434 ± 0.161	0.5266 ± 0.087	0.0283 ± 0.044	0.0339 ± 0.042

Various concentrations of AmpB extending from "100 µg/ml to 0.1 µg/ml" were used. The MIC values for *Candida albicans* cells cultured on two distinct carbon sources, glucose and GlcNAc, were determined. The absorbance measured using a microplate reader at 620nm against different concentrations of AmpB was standardized (mean standard deviation). The MIC values have been highlighted in bold. (WC)* stands for "without culture." [183].

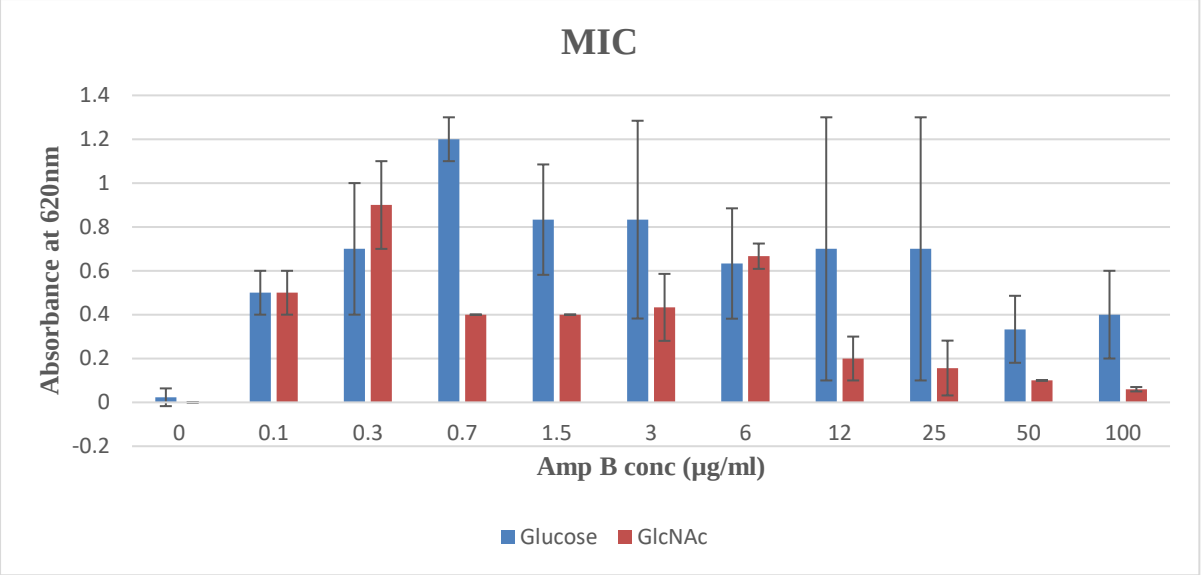


Figure 12: MIC of glucose- and GlcNAc-induced cells. Different concentrations of AmpB extending from "100 µg/ml to 0.1 µg/ml" were used to plot against Absorbance at 620nm. MIC values were calculated with respect to control [Glucose = 1.1, GlcNac = 1]. Absorbance that was listed through a ELISA microplate reader against various concentrations of AmpB was standardized (mean ± standard deviation).

5.5 Metabolite profiling using HPLC, GC-MS, and LC-MS:

We also executed metabolite analysis to evaluate the low molecular weight substances (metabolites) in "yeast and hyphal cells of *C. albicans*." Non-targeted metabolite analysis was done using GC/MS. We performed an HPLC analysis on the material before GC-MS to determine whether the compounds were expressed differently in glucose vs. GlcNAc-grown samples (Figure 11). Usually, there are about 90 peaks resolved in the lysates of cells fed on glucose and GlcNAc. The identification of 23 compounds with a high degree of certainty was followed by extensive categorization (Table 2). According to a GC/MS analysis, the plurality (40%) of the identified metabolites declined and about 20% exhibited rising abundance through GlcNAc-induced morphogenesis. Major metabolites are found in both, such as ascorbic acid (which prevents *C. albicans* from converting from yeast to a hypha) and arachidic acid (which *C. albicans* uses to make extra-cellular prostaglandins and are pivotal for the development of hyphae and host cell infection). Recently, it was discovered that linoleic acid and various fatty acids, like "conjugated linoleic acid (CLA)," block the *Candida albicans* germ tube. Even in

the lack of galactose, UDP-galactose impacts the integrity and morphology of cell walls. Farnesol build-up prevents the transition from yeast to hyphae formation at large cell concentrations. “Ergosterol, a sterol found on the cell membranes of fungus,” and Ethylparaben, an antifungal drug, both functions to preserve the stability of cell membranes. Consequently, these trials proved that the “GlcNAc-induced” cells use the fatty acids for the formation of acetyl-CoA. This leads in the creation of energy (ATP) essential for hyphae and extra-membranous structure development.

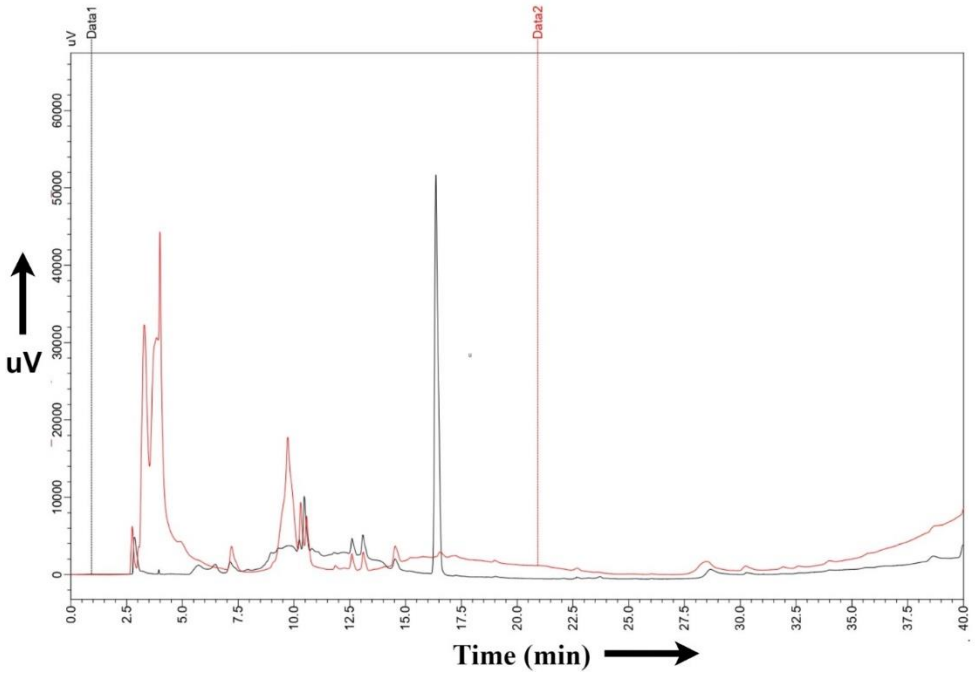


Figure 13: HPLC outcome of “Glucose- and GlcNAc-induced” *Candida albicans* cells. Data 1 (black) represents peaks of metabolites expressed in “N-acetylglucosamine (GlcNAc)-induced cells”, whereas Data 2 (red) represents peaks of metabolites induced in “glucose-induced cells”. The difference in peaks between the two carbon utilisation methods is plainly seen. [183].

Table 2: Concentration of metabolites expressed by "Glucose and GlcNAc-induced" *C. albicans* (SC5314) cells through GC-MS.

METABOLITES	Glucose (Area of peaks)	GlcNAc (Area of peaks)
Acetic acid	75523	66024
Oxalic acid	19379	35533
Formic acid	139232	0
Carbamic acid	0	46075
Carbonic acid	0	24857
Malonic acid	225082	183445
Stearic acid	217183	105159
Trichloroacetic acid	177869	77711
Lignoceric acid	114099	0
Pthalic acid	466065	422575
Erucic acid	272754	0
Oleic Acid	336842	0
Arachidic acid	138511	0
Linoleic acid	183765	0
Myristic acid	212820	0
6-Aminocaproic acid	103442	0
Heneicosane	163744	119053
3,4-Anhydro-d-galactosan	0	10058
Ergosterol	0	144365
Sterols	149667	0
Farnesol	0	95425
3,4-Dihydroxyphenylglycol	2158059	0
Ethylparaben	196123	0
Alanine	0	79622

To check whether there are other metabolites present other than these which may be left out and to validate our result, we performed LC-MS also. We found 15 metabolites in Glucose-induced cells and 17 metabolites in GlcNAc-induced cells as shown (Table 3).

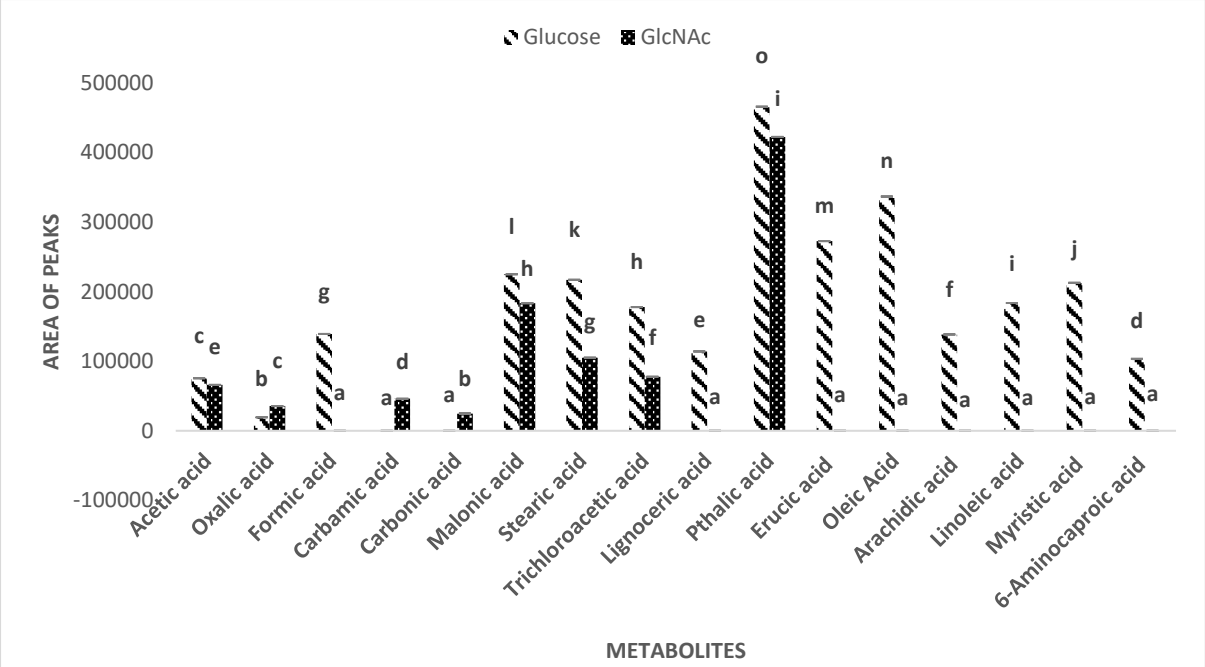


Figure 14: Different metabolite concentrations of Glucose and GlcNAc-induced *C. albicans* cells. Concentration is directly proportional to the area of peaks of that metabolite induced. Significant differences between the bars with various letters may be seen ($p < 0.05$). "one-way ANOVA" and "Tukey's post hoc" analysis between different subjects has been done.

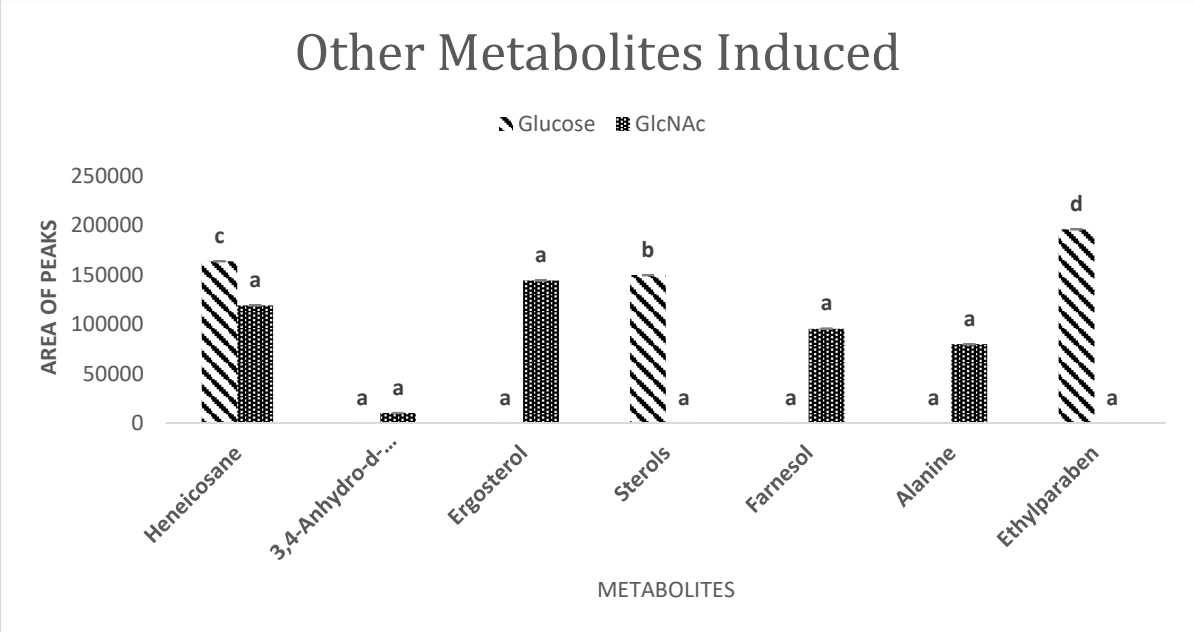


Figure 15: Other metabolite concentrations of Glucose and GlcNAc-induced *C. albicans* cells. Concentration is directly proportional to the area of peaks of that metabolite induced. Significant differences between the bars with various letters may be seen ($p < 0.05$). "one-way ANOVA" and "Tukey's post hoc" analysis between different subjects has been done.

5.6 Metabolites and their function in *Candida albicans*:

5.6.1 Trehalose

Mammals do not contain the nonreducing disaccharide trehalose, which is present in microbes, fungi, plants, and invertebrates. Trehalose functions in yeast as a major glucose store as well as a cellular defence against various stressors [165, 166, 167]. Previous research on *C. albicans* suggested that trehalose plays a particular function in cellular defense against oxidative stress. Trehalose biosynthesis-deficient *C. albicans* were highly susceptible to H_2O_2 exposure and increased intracellular ROS [168, 169]. Tps1 gene disruption in *Candida albicans* impairs hyphal development and reduces infectiousness. Trehalose was found to be critical in the growth of *C. albicans* biofilms and its resilience to antifungals, according to recent research [170]. Both Glucose and GlcNAc-induced cells show almost similar amounts of trehalose present as both are induced in stress conditions.

5.6.2 Lipids Synthesis

In addition to serving as the cells' structural and metabolic elements, lipids also seem to be the cause of *C. albicans'* medication tolerance [171, 172]. In this research, there was a rise in the amounts of several metabolites necessary for the synthesis of lipids, like ethanolamine, inositol, glycerol, and glycerophosphate. The production of phosphatidylethanolamine (PE) begins with ethanolamine. Additionally, it can be utilized to produce choline, a vital “headgroup of phosphatidylcholine (PC).” Inositol is a component for the production of “phosphatidylinositol (PI).” The essential phospholipids in yeast are PE, PC, and PI. Additionally, PI is necessary to produce sphingolipids. It is seen more in GlcNAc-induced cells as hyphae are formed. A new study by Lattif et al. demonstrated the significance of lipids in the development of biofilms and antifungal tolerance [173]. In addition to its function in lipid production, glycerol also functions as a suitable solute that is essential for osmoadaptation. The random intracellular build-up of glycerol in yeast is one distinguishing trait of the osmotic stress defence response.

In addition to its function in the production of lipids, inositol is necessary to produce GPI bonds. GPI production and/or anchoring are critical for “yeast cell viability.” Several proteins present in the “cell wall” and “plasma membrane” implicated in the synthesis and construction of fungal cell walls are GPI attached. About 115 “GPI proteins” that are confined to the “cell wall” or “plasma membrane” are expected to be encoded by *C. albicans* [174]. These GPI

proteins have been found to enact in adhesion, oxidative stress tolerance, and cell wall production and stability [175, 176, 177]. Most of the lipids are enhanced in GlcNAc-induced cells as hyphae and extra membranous structures are formed.

5.6.3 Glutathione metabolism

Antioxidant glutathione is essential for cellular defense against reactive species. Cysteine, glycine, and glutamate are the building blocks needed to make glutathione. Glutathione was transpeptided to create -glutamylamine, which could then be changed into pyroglutamate. This process produces pyroglutamate, which can be changed into glutamate. According to research by "Baek et al.," the production of a "null mutant strain" lacking in "glutathione synthesis" resulted in a rise in intracellular ROS and the onset of "cell death in *C. albicans*," demonstrating the significance of glutathione function to development [178]. Zhu et al. have recently suggested that glutathione depletion has a comparable impact on *C. albicans* cells manifested to farnesol [179]. Noteworthy finding by "González-Párraga et al.," is that "glutathione reductase," an enzyme that converts "glutathione disulfide (GSSG)" to the "sulfhydryl form of glutathione" [180]. Glutathione is found in Glucose-induced cells as in GlcNAc-induced cells it is being utilized to control intracellular ROS.

5.6.4 TCA Cycle, Glycolysis, and Gluconeogenesis

The TCA cycle is an important metabolic pathway in mitochondria that provides energy. The TCA cycle is also a significant metabolic pathway that converts various compounds, including "amino acids", into intermediates that may later be utilised to synthesise amino acids. "Glycolysis, glyoxylate and dicarboxylate metabolism, and fatty acid metabolism" all produce acetyl CoA. NADH is produced as a byproduct of "ethanol fermentation, fatty acid metabolism, and fructose metabolism". These metabolites are produced in "glucose-induced" cells, whereas they are used in GlcNAc-mediated cells; nonetheless, "flavin adenine dinucleotide (FAD)" is a metabolite for "Fatty acid metabolism, Riboflavin metabolism, Citric Acid Cycle, and the Steroid biosynthesis," which is found in GlcNAc-mediated cells. [183].

Citrulline, extracted in Arginine and proline metabolism, and Histidine extracted from its synthesis and beta-Alanine metabolism were also found in GlcNAc-induced *C. albicans* cells.

The metabolic route known as the fructose metabolism pathway involves D-fructose 2,6-bisphosphate, which was found in Glucose-induced cells whereas Fructose 1,6-diphosphate used in Glycolysis I and ethanol fermentation was found in GlcNAc-induced cells.

Biotin affects its metabolism, Fatty acid biosynthesis, and TCA Cycle also helps in *Candida albicans'* colonization and proliferation. It is mostly found in GlcNAc-induced cells.

5.6.5 Other Metabolites

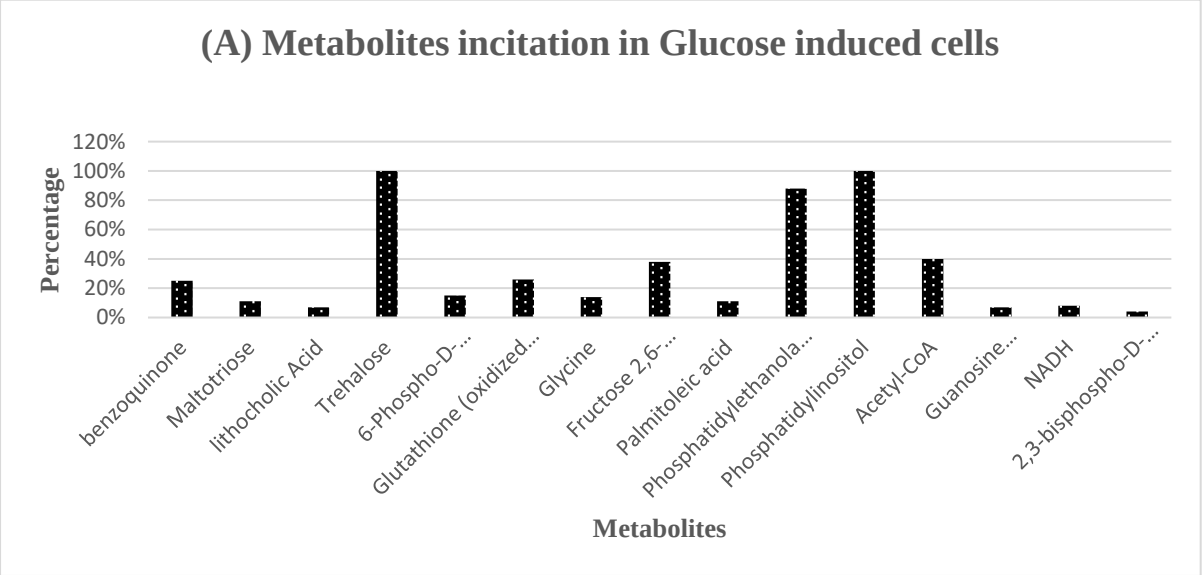
Benzoquinone- By advancing a “two-electron reduction” of quinone molecules, which avoids the generation of harmful semiquinone radicals, flavodoxin-like proteins (Pst3) fight oxidative stress. Pst3 significantly contributed to the development of p-benzoquinone resistance to petite quinone molecules.

Maltotriose- The same genetic processes that control the maltose transcriptional activator in Saccharomyces cerevisiae also control the maltotriose utilization from the permease encoded by AGT1. This function could also be found in *Candida albicans.* It also helps in the metabolism of sucrose and starch.

Table 3: Metabolites and difference in their percentage expressed by Glucose and GlcNAc-induced *C. albicans* (SC5314) cells through LC-MS.

Glucose-induced cells		**GlcNAc-induced cells**	
Metabolites	**Percentage**	**Metabolites**	**Percentage**
Benzoquinone	25%	**D-Glucosamine 6-phosphate**	7%
Maltotriose	11%	**Citrulline**	20%
lithocholic Acid	7%	**L-Histidine**	8%
Trehalose	100%	**Trehalose**	100%
6-Phospho-D-gluconic acid	15%	**6-Phospho-D-gluconic acid**	4%
Glutathione (oxidized form)	26%	**Pyridoxamine 5'-phosphate**	2%
Glycine	14%	**Fatty Acids**	5%
Fructose 2,6-diphosphate	38%	**Fructose 1,6-diphosphate**	4%
Palmitoleic acid	11%	**Taurocholic acid**	4%
Phosphatidylethanolamine	88%	**Phosphatidylethanolamine**	100%
Phosphatidylinositol	100%	**Phosphatidylinositol**	100%
Acetyl-CoA	40%	**5-Phosphorylribose 1-pyrophosphate**	9%
Guanosine diphosphate mannose	7%	**Thiamine pyrophosphate**	18%
NADH	8%	**Adenosine**	2%

2,3-bisphospho-D-glyceric acid	4%	**Flavin adenine dinucleotide**	9%
		Oleic acid	14%
		Biotin	9%



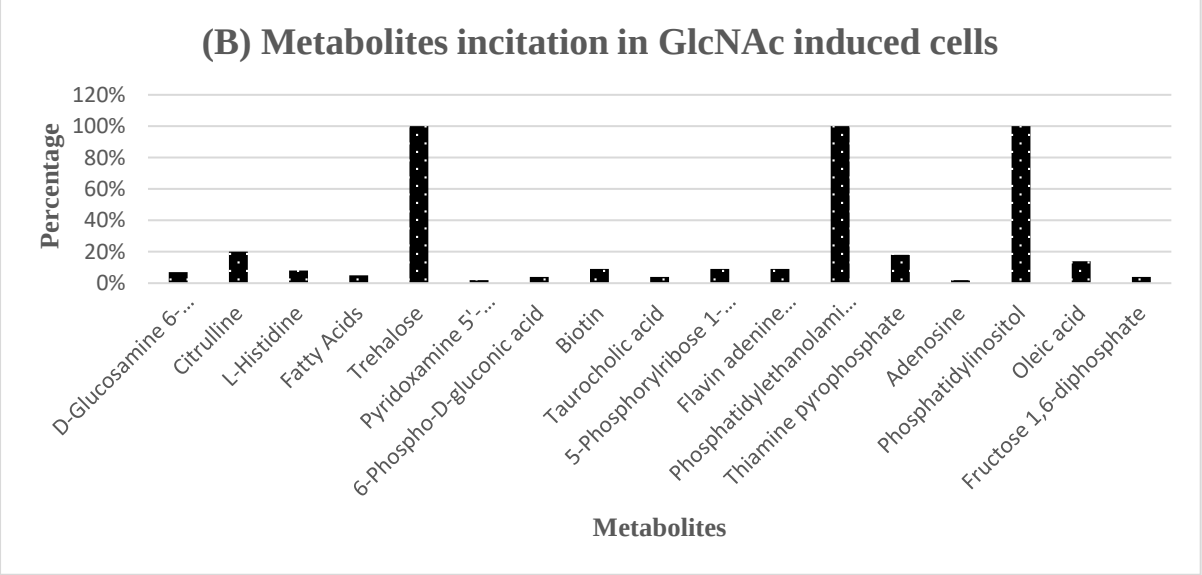


Figure 16: Metabolite concentrations of Glucose and GlcNAc-induced *C. albicans* cells analysed trough LC-MS. The percentage is directly proportional to the area of peaks of that metabolite induced. (A) Corresponds to the data of Glucose-induced cells. (B) Corresponds to the data of GlcNAc-induced cells.

5.7 RNA expression of different virulence and hyphal-specific genes:

ACT1: Actin, a highly conserved protein that helps maintain the cytoskeleton's structural integrity, is produced by the ACT1 gene.

NGT1: “N-acetylglucosamine (GlcNAc)-specific transporter”; function in GlcNAc driven hyphal development; localizes to “plasma membrane”; triggered by GlcNAc, “macrophage engulfment”; main facilitator superfamily; having 12 transmembrane proteins.

DAC1: N-acetylglucosamine-6-phosphate (GlcNAcP) deacetylase that transforms GlcNAc-6-phosphate to Glucosamine-6-phosphate, GlcNAc-induced gene, and protein, N-acetylglucosamine utilization, and wild-type hyphal development and virulence in mouse systemic infection.

NAG1: Normal hyphal development and mouse pathogenicity depend on the enzyme glucosamine-6-phosphate deaminase, which converts glucosamine 6-P to Glucose 6-P in a reversible process in vitro.

HWP1: At the MTLa side of the conjugation tube, the hyphal cell wall protein is an opaque, a-specific, alpha-factor driven substrate. virulence made more difficult by URA3 impacts; Induced by spider biofilms in RPMI a/a biofilms, Bcr1 repression.

ECE1: Candidalysin, a cytolytic peptide toxin necessary for mucosal infection, forms holes in epithelial cells by assembling into polymers; a particular protein for hyphae.

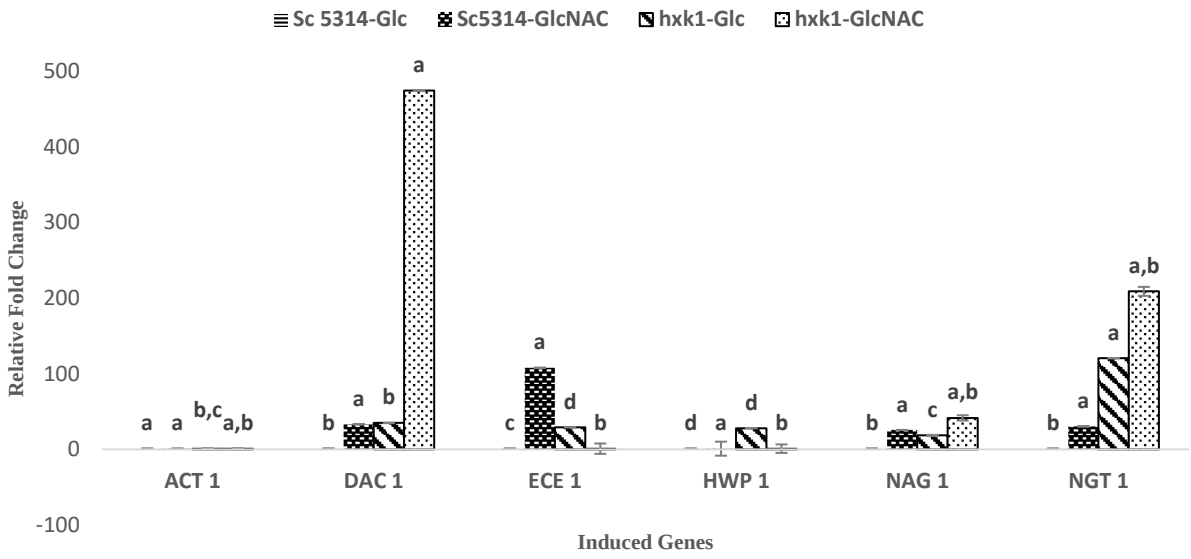


Figure 17: Relative fold change in expression of virulence and hyphal-specific genes in SC5314 Glucose, SC5314 GlcNAc, ΔHXK1-Glucose, and ΔHXK1- GlcNAc induced *Candida albicans*. SC5314 Glucose is taken as the base and accordingly, the fold change is described. ACT1 is a housekeeping gene and is used as a reference/control as its expression levels do not change. Significant differences between the bars with various letters may be seen ($p < 0.05$). "one-way ANOVA" and "Tukey's post hoc" analysis between different subjects.

DAC1 is more expressed in ΔHXK1-GlcNAc induced *C. albicans* cells but not utilized as the mutated strain cannot convert extracellular GlcNAc to GlcNAc-6-phosphate due to the unavailability of the kinase HXK1. But Dac1 is expressed and utilized by both SC5314 GlcNAc induced as well as ΔHXK1-Glucose induced cells. ECE1 is more expressed in SC5314 GlcNAc-induced *C. albicans* cells as these are more virulent. ΔHXK1-Glucose-induced cells express Ece1 in marginal amounts as the virulent factors are limited. HWP is expressed in only ΔHXK1-Glucose-induced cells as in all cases that form hyphae the HWP converts into hyphal-specific proteins. NGT1, N-acetylglucosamine (GlcNAc)-specific transporter is more expressed in ΔHXK1-GlcNAc induced *C. albicans* cells as it is processed due to induction of GlcNAc but not utilized as ΔHXK1 strain cannot convert extracellular GlcNAc to GlcNAc-6-phosphate.

6. Summary and Conclusion

GlcNAc is utilized by the heterogeneity of organisms like bacteria, fungi, and humans, as a nutrition and signalling molecule. It provides a constant supply of carbon, both in the host animal and in the surrounding environment. *C. albicans* is able to utilize "GlcNAc as a carbon source," thanks to its catabolic route for the molecule. GlcNAc is a food source and a "signaling molecule" that can trigger filamentous protrusion and a change in colour from "white to opaque in *C. albicans*." Since this dual morphological transformation has been significantly studied in this fungus, "*C. albicans*" is a great model organism to further research the fundamental "molecular mechanisms of GlcNAc sensing" and usage. GlcNAc may influence *C. albicans* signalling in one of two ways. First, it can work as a signalling molecule even in the absence of catabolism. Second, catabolism can make the extracellular environment alkaline, which further encourages hypha production. In *C. albicans*, GlcNAc also promotes the translation of genes involved in virulence, implying that it may influence pathogenesis.

In "mucocutaneous infection systems" like "oropharyngeal candidiasis," yeasts are analogous with commensalism; nevertheless, filamentous forms of the yeast "hyphae and pseudohyphae" relate to tissue invasion and damage. Disseminated sickness, including abscesses inside the host's internal organs, appears to be influenced by yeasts, pseudohyphae, and hyphae. Evidence suggests that the amino sugar GlcNAc functions as a superb "signalling molecule" in *C. albicans*, modulating a variety of dynamic cellular processes like the organism's own "sugar metabolism," the "morphogenetic transition" (the switch from "yeast to hyphae" and from "white to opaque"), "GICD - GlcNAc induced cell death", virulence, and interspecies communication. Accordingly, it is thought that "GlcNAc signalling" and its metabolism give *C. albicans* an adaptive edge so that it can react quickly and properly to the "host niche" environmental circumstances for maximizing nutrition uptake and out-competing other bacteria and fungi. Deciphering the "molecular pathways of GlcNAc signalling" in this fungus pathogen is therefore crucial. So, we investigated the effects of various carbon sources on *Candida albicans* cells and concluded that various carbon sources may alter the shape of the organism from "yeast to hyphae" and can also trigger various metabolites that aid in hyphae production and pathogenesis.

While performing experiments regarding the morphological change, we concluded that "Glucose-induced" cells are round and distinct in the colony, and "GlcNAc-grown" cells are

elongated, flat and create a smear. We discovered that GlcNAc-treated cells demonstrated hyphal development in contrast to the Glucose control cells performing SEM analysis. We can differentiate because, according to the study, GlcNAc-induced cells are larger than usual glucose-induced cells (1.23 mm x 0.67 mm vs. 9.60 mm x 3.80 mm). This morphogenetic transition is a crucial step in the "virulence and pathogenesis" of *C. albicans*. The yeast-to-hyphae transition is synchronized by multiple "signaling pathways," and GlcNAc has been identified as a potent inducer of this transition. When *Candida albicans* are exposed to GlcNAc, it triggers a series of events that lead to the activation of key "transcription factors" and the expression of specific "genes involved in filamentation." The major pathway involved in the morphogenetic switch is the "cAMP-PKA pathway." GlcNAc increases the intracellular quantity of "cyclic adenosine monophosphate (cAMP)", which trigger "protein kinase A (PKA)" and promotes the yeast-to-hyphae transition. Activation of PKA leads to the phosphorylation of downstream targets, including transcription factors like Efg1 and Ras1, which are involved in filamentation. GlcNAc can also modulate other signaling pathways, such as the "mitogen-activated protein kinase (MAPK) pathway." It has been shown to operate the "Cek1- MAPK pathway", which is essential for the expression of "hypha-specific genes". Additionally, GlcNAc can influence the expression and activity of other transcription factors, such as Tec1 and Ndt80, which regulate filamentation-related genes. The induction of morphogenetic changes by GlcNAc is important for the virulence of *Candida albicans*. The hyphal form allows *C. albicans* to invade host tissues, penetrate epithelial barriers, and form biofilms. These traits are associated with increased resistance to host immune defenses and the ability to cause tissue damage.

One of the primary effects of GlcNAc on *Candida albicans* is the activation of the "cAMP-PKA pathway". Activation of the cAMP-PKA pathway leads to a variety of metabolic changes, including the upregulation of genes involved in chitin synthesis and cell wall remodeling. GlcNAc can also influence the expression of genes involved in carbon metabolism and energy production. Studies have shown that GlcNAc can enhance the expression of genes encoding enzymes involved in glycolysis, the TCA cycle, and oxidative phosphorylation. This suggests that GlcNAc may provide an additional carbon source for energy production in *C. albicans*. Furthermore, GlcNAc can impact the expression of virulence factors. It has been shown to increase the expression of adhesins, which are molecules that facilitate the attachment of Candida cells to host tissues. GlcNAc also promotes the yeast-to-hyphae transition, a crucial step in virulence. In summary, N-Acetylglucosamine can induce metabolic changes in *C. albicans* by activating the cAMP-PKA pathway, altering carbon metabolism and energy

production, and influencing the expression of virulence factors. These changes contribute to the adaptation and virulence in various host environments. Following the certain identification of 23 compounds, there was a thorough classification process. A GC/MS study revealed that during "GlcNAc-induced morphogenesis", most (40%) of the identified metabolites dropped and roughly 20% showed increasing abundance. Major metabolites were present in both, including arachidic acid and lignoceric acid, which *C. albicans* utilize to produce extra-cellular prostaglandins that are essential for the growth of hyphae and host cell infection respectively. Additionally, lignoceric acid has been demonstrated to possess antifungal qualities, suggesting that it may find application in the creation of novel medications intended to address fungal diseases.

The exposure of *Candida albicans* to N-Acetylglucosamine (GlcNAc) can lead to the upregulation of several genes involved in various cellular processes. Here are some examples of genes that have been found to be incited in GlcNAc-induced *C. albicans*:

1. NAG genes: NAG1, NAG2, NAG3 - These genes encode enzymes involved in GlcNAc metabolism. They are responsible for the conversion of N-Acetylglucosamine to fructose-6-phosphate, which can afterwards go via the glycolytic pathway.
2. EFG1 - This gene encodes a "transcription factor Efg1", which is a crucial regulator of morphogenesis in *C. albicans*. GlcNAc induces the "expression of EFG1," leading to the activation of filamentation and the transition from the "yeast to hyphal form."
3. RAS1 - The RAS1 gene encodes a small GTPase involved in signal transduction pathways. GlcNAc can activate Ras1, which is required for the "yeast-to-hyphae" transition and "virulence in *C. albicans*."
4. TEC1 - Tec1 is a "transcription factor" that plays a role in regulating the expression of "hypha-specific genes." GlcNAc has been manifested to induce the expression of TEC1, contributing to filamentation and the development of hyphal structures.
5. HWP1 - The HWP1 gene encodes a "cell wall protein" known as "Hyphal Wall Protein 1." GlcNAc can induce the expression of HWP1, which is associated with hyphal adhesion and biofilm formation.
6. ALS3 - ALS3 encodes an adhesin protein called Als3, which mediates adherence of *Candida albicans* to host tissues. GlcNAc has been shown to upregulate ALS3 expression, promoting host tissue adhesion and colonization.

7. SAP genes: SAP4, SAP5, SAP6 - These genes encode secreted aspartyl proteases, which are important virulence factors in *C. albicans*. GlcNAc can induce the expression of SAP genes, contributing to tissue invasion and nutrient acquisition.

These are just a few examples of the genes that can be incited in N-Acetylglucosamine-induced *Candida albicans*. The precise gene expression changes may vary depending on the experimental conditions and the specific genetic background of the Candida strain being studied. We have used virulence and hyphal-specific genes for RT-RT-PCR to differentiate between Glucose and GlcNAc-induced *C. albicans* (SC5314). ΔHXK1 strains were also used to check the working capacity of the GlcNAc catabolic pathway. ACT1 gene was used as a housekeeping gene to normalize the general functioning of cells. Although DAC1 is more abundant in ΔHXK1-GlcNAc-induced *C. albicans* cells, it is not used since the mutant strain cannot convert extracellular GlcNAc to GlcNAc-6-phosphate because HXK1 is not present. However, Dac1 is produced and used by cells that have been stimulated by SC5314 GlcNAc as well as ΔHXK1-Glucose. Due to the increased virulence of SC5314 GlcNAc-induced *C. albicans* cells, ECE1 is highly expressed in these cells. Ece1 is only moderately expressed in ΔHXK1-Glucose-induced cells since there are not many pathogenic factors present. Only ΔHXK1-Glucose-induced cells produce HWP because in all instances when hyphae develop, HWP transforms into hyphal-specific proteins. NGT1, a transporter that is specific to N-acetylglucosamine (GlcNAc), is highly expressed in HXK1-induced GlcNAc-induced *C. albicans* cells because this strain is unable to convert extracellular GlcNAc to GlcNAc-6-phosphate.

We have literally proved that the availability and utilization of different carbon sources can influence the virulence of *C. albicans*. Studies have shown that when *C. albicans* cells are grown in the presence of GlcNAc, they exhibit increased virulence compared to cells grown in glucose. GlcNAc-induced cells show enhanced adhesion to host cells, increased biofilm formation, and greater resistance to antifungal agents. One of the main reasons for this difference in virulence is the induction of specific virulence genes by GlcNAc. GlcNAc induces the expression of genes implicated in adhesion, invasion, and “biofilm formation,” which are important for *C. albicans* to establish infections. These genes include ALS3, HWP1, ECE1, and others. Our findings show distinct metabolic variations between yeast and hyphae, as well as characteristic metabolic rearrangements during filamentation. On the metabolic side, we discovered that the *C. albicans* metabolomes were mostly grouped by growing medium, proposing that, unlike the transcriptome, they had a higher influence on “cellular metabolism”

than “morpho- or genotype”. This is backed up by the lack of a compatible “genotype-independent” association between “morphotype and metabolome”. Furthermore, GlcNAc also affects the morphological transformation of *C. albicans*. It promotes the “yeast-to-hyphae” transition, which is associated with increased virulence. Hyphae are filamentous structures that can penetrate host tissues and evade the immune system more effectively than yeast cells. In contrast, when *C. albicans* cells are grown in glucose, they exhibit reduced virulence traits. Glucose represses the expression of certain virulence genes and inhibits the yeast-to-hyphae transition. As a result, glucose-induced cells are generally less adherent, have reduced biofilm formation, and are more susceptible to antifungal treatments. It is important to note that the relationship between carbon source utilization and virulence in *C. albicans* can be complex and influenced by various factors. While GlcNAc-induced cells tend to be more virulent than glucose-induced cells, the specific conditions and host environment can also modulate these effects. Additionally, *C. albicans* virulence is a multifactorial process, involving various other factors such as host immune responses and interactions with other microorganisms. Finally, we are sure that more research based on the results given here will illuminate the metabolic characteristics of hyphae, eventually leading to a better knowledge of fungal physiology in general.

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