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Optimization of isolation of RNA from yeasts for the purpose of transcription analysis

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Optimalizace izolace RNA z kvasinek pro následné transkripční analýzy

Bc. Valin Lay

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Teoretická část:

Obecný popis patogenních a nepatogenních kvasinek (např. *Saccharomyces cerevisiae, Candida albicans, Candida parapsilosis* a dalších), využití transkriptomických analýz pro studium genové regulace v kvasinkách. Charakteristika metody real-time PCR a jejího využití.

Experimentální část:

1. Kultivace různých druhů kvasinek (např. *Saccharomyces cerevisiae, C. albicans* a *C. parapsilosis*).
2. Izolace RNA pomocí různých izolačních metod/kitů.
3. Elektroforetická analýza získané RNA.

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V Pardubicích dne 28. února 2023

Declaration:

The diploma thesis titled **Optimization of isolation of RNA from yeasts for the purpose of transcription analysis** is my own work and research. It has been written by me and has not been submitted for any previous degree program. All literary sources and information I have used are listed in the list of used literature.

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TITLE:

Optimization of isolation of RNA from yeasts for the purpose of transcription analysis

ANNOTATION:

The diploma thesis deals with finding the optimal way to isolate RNA from different species of yeasts in the maximal possible amount and purity, especially from the non-pathogenic yeast (*Saccharomyces cerevisiae*) and pathogenic yeasts (*Candida albicans* and *Candida parapsilosis*). The theoretical section describes the basic information about these yeasts and utilization of transcriptomic analyses to study of their gene regulation. The characteristics of the PCR method, as well as its use for RNA transcription, are also mentioned.

The practical section in the second part of the thesis contains partial steps of cultivation of different yeast species, RNA isolation using two different isolation methods (TRIzol reagent-phenol chloroform extraction and Quick-RNA Viral Kit), and the electrophoretic analysis of the obtained RNA.

KEY WORDS:

yeasts, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida parapsilosis*, RNA isolation, transcription analysis, PCR

NÁZEV:

Optimalizace izolace RNA z kvasinek pro následné transkripční analýzy

ANOTACE:

Diplomová práce se zabývá nalezením optimálního způsobu izolace RNA z různých druhů kvasinek v maximálním možném množství a čistotě, zejména z nepatogenních kvasinek (*Saccharomyces cerevisiae*) a patogenních kvasinek (*Candida albicans* a *Candida parapsilosis*). V teoretické části jsou popsány základní informace o těchto kvasinkách a využití transkriptomických analýz ke studiu jejich genové regulace. Zmíněna je také charakteristika metody PCR a její použití pro transkripci RNA.

Praktická část ve druhé části práce obsahuje dílčí kroky kultivace různých druhů kvasinek, izolaci RNA pomocí dvou různých izolačních metod (extrakční činidlo TRIzol-fenol chloroform a Quick-RNA Viral Kit) a elektroforetickou analýzu získané RNA.

KLÍČOVÁ SLOVA:

kvasinky, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida parapsilosis*, izolace RNA, transkripční analýza, PCR

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LIST OF ABBREVIATIONS

- **1n** - haploids
- **2n** - diploids
- **BC** – before Christ
- **BSC** - biological species concept
- **bZIP** - basic leucine zipper
- **cDNA** - complementary DNA
- **DNA –** deoxyribonucleic acid
- **dPCR** - digital PCR
- **EPSs** - extracellular polymer substances
- **ER** – endoplasmic reticulum
- **eRNA** - enhancer RNA
- **gDNA** - genomic DNA
- **GITC** - phenol-guanidine isothiocyanate
- **KCl** - potassium chloride
- **lincRNA**- long intergenic ncRNAs
- **$MgCl_2$** - magnesium chloride
- **miRs** - microRNAs
- **mRNA** - messenger RNA
- **NAC** - non-*Candid*a *albicans* species
- **ncRNA** - non-coding RNA
- **NE** - nuclear envelope
- **NHR** - nuclear hormone receptor
- **NNISS** - national nosocomial infections surveillance system
- **nPCR** - nested PCR
- **NPCs** - nuclear pore complexes
- **PCR** - polymerase chain reaction
- **Pol I** - polymerase I
- **qPCR** - real-time (quantitative) PCR

- **rDNA** - ribosomal DNA
- **RNA –** ribonucleic acid
- **RNA-Seq** - transcriptome sequencing
- **RNase** – ribonuclease
- **rRNA** - ribosomal RNA
- **RT** – room temperature
- **RT-PCR** - reverse transcription PCR
- **RT-qPCR** - reverse transcription-quantitative PCR
- **siRNA** - small interfering RNA
- **snoRNA** - small nucleolar RNA
- **snRNA** - small nuclear RNA
- **SPB** - spindle pole body
- **TBE** - Tris-borate-EDTA
- **TF** - transcription factor
- **TRIzol** – monophasic solution of phenol and guanidinium isothiocyanate
- **tRNA** - transfer RNA
- **YNB** - yeast nitrogen base
- **ZF** - zinc finger

INTRODUCTION

Yeast is a single-cell eukaryotic organism in the family of fungi. This biosystem has recently been the subject of extensive research and has been one of the best models for studying gene sequences and conducting comparative genomic studies (Heil, 2021). *Candida albicans* is the most common human pathogen, which predominantly causes different fungal infections worldwide. This yeast typically exists on human skin, and inside the body such as the mouth, throat, intestine, and vagina. It normally lives as a harmless pathogen to healthy hosts, but it develops into a serious health problem for immunocompromised individuals. Similar to *Candida albicans*, the opportunistic yeast *Candida parapsilosis* is known as the second most widely isolated *Candida* species from bloodstream infections and can lead to both superficial and invasive illnesses in humans. The incidence related to this pathogen has been noticeably increasing. The best model organism for the study is *Saccharomyces cerevisiae* or also known as the baker's yeast. This yeast genome was fully sequenced for the first time in eukaryotes in 1996. Thanks to its basic genome along with its rapid growth rate, it has emerged as a useful model for a variety of research studies (Remziye, 2012). The yeast *C. albicans* and *C. parapsilosis* share similar molecular mechanisms with *S. cerevisiae*. However, they are very different from one another and, in addition, haven't extensively undergone study. Despite the fact that the genomes of *C. albicans* and *C. parapsilosis* have been already sequenced, only a small portion of the genes and the related proteins have been thoroughly characterized through experiments.

Transcriptomic analysis has grown in popularity recently and has become a key component in biological study (Geoghegan et al., 2020). Obtaining high quality and quantity of RNA is required for researching gene expression and having an impact on how accurately gene expression is assessed (Rodríguez et al., 2018; Tavares et al., 2011). Yeast RNA isolation has been very challenging. Especially, for the yeast cells that contain an extensive amount of polysaccharides and proteins (Remziye, 2012). There are various techniques and commercial kits available these days. For example, most of the recent and effective ways to extract RNA from the yeast *S. cerevisiae* are water-base techniques, RNAsnap^{TM}, acid hot-phenol extraction, and one-step hot formamide extraction (Lee et al., 2019). Advanced techniques like microarray, and real-time reverse transcription-PCR (qRT-PCR) are always competent PCR methods to yield both qualitative and quantitative amounts of RNA.

The experimental part of the thesis is mainly focused on finding an optimal method of RNA isolation from three different yeast species, including *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida parapsilosis*. The aim of these experiments was to find a suitable method for RNA isolation that obtain the most sufficiently pure in both quantity and quality amount of RNA by comparing the method TRIzol$^{@}$ reagent-phenol chloroform extraction and Quick-RNA^{TM} Viral Kit. The obtained RNA was compared by further analyzing using agarose gel electrophoresis.

1 THEORETICAL SECTION

1.1 The yeast

1.1.1 The history of yeast

Yeast is a biosystem that has recently been the subject of extensive research. This organism is one of the best models for studying gene sequences and conducting comparative genomic studies. The analysis of metabolic pathways and biotechnological applications is made possible by molecular investigations and gene sequencing connected to phylogeny (Lahir, 2022). Yeasts benefit humanity by being widely used in the production of foods, wine, beer, and a variety of biochemicals. Yeasts also cause food and beverage spoilage and are medically important (Boekhout & Kurtzman, 1996).

Even the fact that living microbes existed was up for debate at the beginning of the nineteenth century. One of the first microbes to be scientifically studied was yeast, aside from the slightly larger molds. This was most likely due to the fact that its cells are significantly larger than those of the majority of bacteria and that the alcoholic fermentation industries provided a sizable amount of financial support (Barnett, 2003).

Only a few of the estimated 700 yeast species that have been identified so far are well-known. Since only a small number of natural habitats have been thoroughly examined for yeast species, we can assume that many more species are still undiscovered. Considering how frequently yeasts are used in both conventional and modern biotechnology, the search for new species should result in the development of additional novel technologies (Boekhout & Kurtzman, 1996).

Earlier, the fermentation of fruit juice, like those of apple and pear to produce cider, grapes to obtain wine, or grains to make beer, and so on for any kind of alcoholic beverages, have been carried out by naturally occurring microorganisms present (Pulcini et al., 2022).

The earliest archaeological proof of the development of the technology for making fermented beverages from rice, honey, and fruit was discovered in China in ancient tombs dating back to more than 7000 BC. Data on the use of yeast in wine fermentation processes in ancient Egypt date back to 3150 BC, while chemical analysis data confirming the start of winemaking date back to 5400 BC. Technology for making fermented beverages originated in Egypt and Mesopotamia, and it later traveled to Europe and the New World (Eldarov et al., 2016).

Pasteur established the role of yeast in alcoholic fermentation in 1860. The first pure yeast culture was discovered by Emil Christian Hansen of the Carlsberg Laboratory in the early 1980s, and it was later used as an inoculum for the fermentation of wine essentials. Amazingly, starter cultures are now used almost exclusively in commercial wine production worldwide. This practice only started to spread effectively in the middle of the 20th century. Utilizing carefully chosen commercial yeast strains has significantly increased the wine fermentation process' controllability and dependability, reduced the variability of the wine's microbial composition, and significantly improved wine quality in recent years (Eldarov et al., 2016).

1.1.2 Natural yeast habitats

Yeasts are eukaryotic microorganisms that inhabit a wide range of ecological niches (Eldarov et al., 2016). Although yeasts are not as common as bacteria in the environment, they are most commonly found in water, soil, animals, insects, air, and on the surfaces of plants and fruits (Table 1) (Eldarov et al., 2016; Walker, 2009). Despite a few species being found in commensal or parasitic relationships with animals, yeast is most commonly found in plant tissues like leaves, flowers, and fruits. There are a few yeasts that are opportunistic human pathogens, most noticeably *Candida albicans* (Walker, 2009).

Since yeasts actively participate in the fermentation process and directly affect how ripe fruit decomposes, the latter habitat may currently be the most interesting habitat. Given the right nutrients and substrates, yeasts can successfully carry out their metabolism and fermentation activity in this naturalistic way (Lahir, 2022; Eldarov et al., 2016). Compared to other microorganisms like lactic acid bacteria, yeasts are not particularly nutritionally demanding. The presence of fundamental substances like fermentable sugars, amino acids, vitamins, minerals, and oxygen, however, promotes their growth (Eldarov et al., 2016).

Many different types of yeast can be isolated from specialized or harsh environments, which include those that have low oxygen availability, low temperature, and low water potential for example high concentrations of sugar or salt (Walker, 2009). Extreme environments that support yeasts include stratospheric air, hot springs, cold and deep seas, polar regions, glaciers, deserts, acidic and alkaline habitats, dry rocks, and more. It can be both temporary and permanent, and their definitions have evolved over time (Segal-Kischinevzky et al., 2022).

As primarily free-living decomposers, yeasts speed up a number of fundamental ecological processes, including the breakdown of organic composition, the production of biomass, participation in the nitrogen and sulfur cycles, and the mineralization of nutrients. Yeasts engage in a variety of ecological interactions with other organisms, including fungi, animals, algae as well as plants as pathogens, mutualists, parasites, and many others. They can disperse spores over long distances, even across continents, via wind, water, and vectors. They can adapt to a wide range of biomes, such as those characterized by acidic, alkaline, heat, extreme cold, dryness, osmolarity, toxicity, salinity, or UV radiation conditions alone or in combination. This is made possible by their physiological and metabolic capacities (Segal-Kischinevzky et al., 2022).

Habitat	Description
Animal	Several non-pathogenic yeasts are associated with the intestinal tract and skin of warm-blooded animals;yeasts (*e.g.*, *Candida albicans*) are opportunistic pathogen to humans and animals; yeasts are commensally associated with insects acting as important vectors in the natural distribution
Atmosphere	A few viable yeast cells may be expected per cubic meter of air. Generally, *Cryptococcus*, *Debaryomyces* spp., *Rhodotorula*, and *Sporobolomyces* are dispersed by air from layers above soil surfaces
Built Environment	Yeasts are fairly ubiquitous in buildings. *e.g.*, *Aureobasidium pullulans* is common on damp household wallpaper and *S. cerevisiae* is readily isolated from surfaces in wineries
Plants	Interface between soluble nutrients of plants and the septic world are common niches for yeasts; spread of yeasts on the phyllosphere is aided by insects. The presence of some organic compounds on the surface and decomposing areas creates conditions favorable for growth of yeasts
Soil	Soil may only be a reservoir for the long-term survival of yeast, rather than a habitat for growth. Yeasts are ubiquitous in cultivated soils (nearly 10 000 cells/g of soil) and are found only in the upper, aerobic soil layers (10–15cm). *Lipomyces* and *Schwanniomyces* are isolated exclusively from soil
Water	Yeasts predominate in surface layers of fresh and salt waters, but are not present in great numbers (nearly 1000 cells/L). Most aquatic yeast isolates are of red pigmented genera (Rhodotorula). The species *Debaryomyces hansenii* is a halotolerant yeast that can grow in nearly saturated brine solutions

Table 1: Natural yeast habitats (Monroy Salazar et al., 2016)

1.1.3 General yeast morphology

Cell morphology, physiology, immunological, and molecular biological methods serve as the foundation for their identification. The physical and chemical changes that take place during a yeast cell's growth determine its generalized cell structure (Lahir, 2022). In terms of cell size, shape, and color, yeast cells display a great deal of diversity. Heterogeneity in morphology can be seen in even a single cell from a pure strain of a single species. By changing the physical or chemical conditions at growth, significant changes in individual cell morphology will also be brought about (Feldmann, 2012).

The size of yeast cells varies greatly; while some species can grow to lengths of 20–50 mm, others may only be 2-3 mm long. With a range of 1 to 10 mm, cell width is more consistent (Feldmann, 2012). They resemble septate hyphae and can be ovoid, ellipsoid, hemispherical, filamentous, or occasionally cylindrical in shape (Lahir, 2022). Numerous yeast species have ellipsoidal or ovoid cells as their cell shape. Some have cylindrical bodies with hemispherical ends, like the *Schizosaccharomyces*. For instance, *Yarrowia lipolytica* and *Candida albicans* are primarily filamentous organisms with pseudohyphae and septate hyphae. Additionally, yeast can also be found in elongated or spherical forms such as the *Debaryomyces* species (Feldmann, 2012).

1.1.4 General description of the yeast cell

The cell envelope of the budding yeast controls the osmotic and permeability characteristics of the cell and makes up about 15% of the total cell volume. The cytosol of yeast is encircled by the cell wall, periplasm, and plasma membrane when viewed from the inside out (Feldmann, 2012) (Figure. 1).

The cell wall of *S. cerevisiae* is a dynamic cell organelle in both structural and functional terms. It has an elastic and strong structure that keeps yeast cells in their proper shape and size while also maintaining their unique functional mechanical strength, osmotic pressure, physical protection, and resistance to other types of stresses (Klis et al., 2002; Lahir, 2022). The inner layer of the wall is mainly accountable for mechanical strength and also serves as an adhesion site for the proteins that make up the outer layer of the wall (Klis et al., 2002). The outer shell of the yeast cell wall is firm and has a tough layer with a rigid structure about 100 – 200 nm thick (Feldmann, 2012; Lahir, 2022; Lipke & Ovalle, 1998).

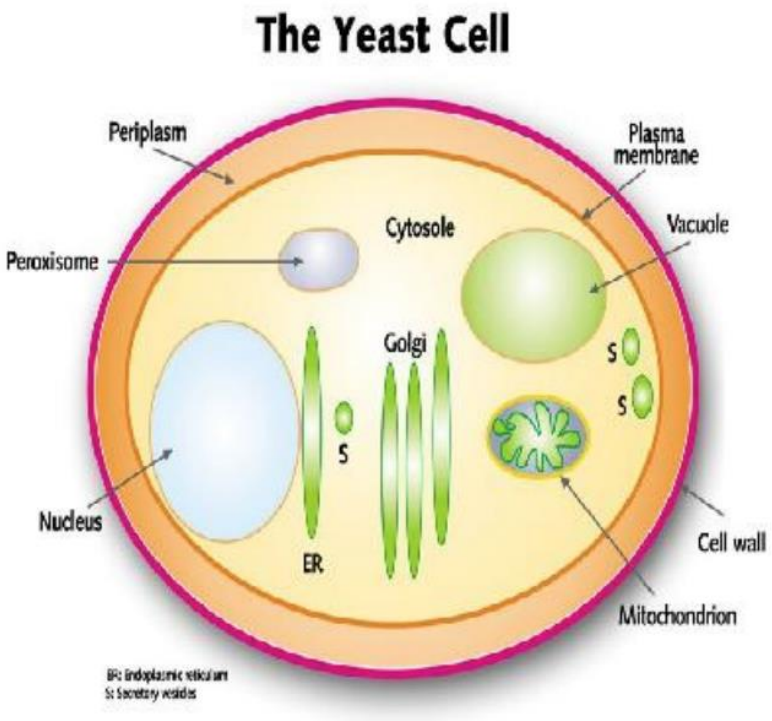

Figure 1: Yeast cell (Monroy Salazar et al., 2016)

The periplasmic space is an extremely thin space measuring 35–45 A, enclosed by the plasma membrane on the inside and the cell wall on the outside. Mostly proteins like invertase and acid phosphatase, which catabolize substrates that do not cross the plasma membrane, are secreted proteins that cannot pass through the cell wall that is found in this space.

A plasma membrane encloses every cell and serves as the barrier separating it from its surroundings. The cell envelope of *Saccharomyces cerevisiae* which consists solely of the plasma membrane and cell wall takes up about 15% of the total volume of the cell (Ferraz et al., 2021). It has a thickness of about 7.5 nm. It occasionally invades the cytoplasm, causing protrusions (Lahir, 2022; Feldmann, 2012). Polar lipids and proteins are mixed together and control the membrane's structure through their interactions.

The cytoplasm is a colloidal, aqueous solution with a pH of 5.2 and contains molecules with low and intermediate molecular weights proteins, glycogen, and other soluble macromolecules. The

inclusions that give the cytoplasm of yeast cells the appearance of being in suspension include larger macromolecular components like ribosomes, proteasomes, or lipid particles. The structure of yeast cytoplasm is extremely dynamic and constantly changing.

Many scientists have used yeast mitochondria as a model system for studying mitochondrial structure, function, biogenesis, bioenergetics, and apoptosis for a very long time. In addition to sharing similarities with these higher eukaryotic organelles, yeast mitochondria are crucial for understanding how fermentation works (Lahir, 2022; Sickmann et al., 2003). Both types (outer and inner membrane) of lipid bilayers surround yeast mitochondria, just like they do in mammals.

The largest membrane-bound organelle in a eukaryotic cell is the endoplasmic reticulum (ER), which is a cisternal space that may take up to 10% of the cell's volume (Austriaco, 2012; Feldmann, 2012). The yeast ER membrane is made up of a lipid double layer that is strongly connected to the nuclear membrane and contains integral proteins (Kim et al. 2019; Feldmann, 2012).

The Golgi complex, also known as the Golgi apparatus is primarily composed of membranous stacks that are distributed throughout the cell or stacked parallel to one another.

The yeast nucleus serves as an excellent model system for comprehending structure-function relationships within one of the most complex compartments in cells. The nucleus is extremely well-organized in terms of both chromosomal sequences and enzymatic activities, similar to those of other eukaryotic species (Blumenberg, 2019). The essential genetic code of an organism is stored in and expressed by the cell nucleus, which also controls chromosome expression, duplication, repair, and segregation as well as messenger and ribosomal RNA processing and export (Taddei & Gasser, 2012).

The nuclear structure is a nearly spherical organelle with a diameter of 1.5 mm, located either in the center or slightly off-center of the cell. The ER membrane and the outer nuclear membrane are essentially one membrane. The yeast nuclear membrane is not resolved during mitosis, in contrast to the majority of eukaryotic cells, whereas it disintegrates in higher eukaryotic cells. A spindle pole body (SPB) that the nucleus carries on its exterior acts as an anchor for cytosolic microtubules as well as for continuous and discontinuous microtubules that cross the nucleus. The SPB is duplicated during mitosis, and this apparatus is responsible for directing the movement of the duplicated chromosomes into the mother and daughter cells prior to cell division (Feldmann, 2012).

The natural channels for mediating cargo transport are nuclear pore complexes (NPCs), which are about 50–100 nm in diameter. NPCs go through significant changes as yeasts age and exchange components between the nucleus and cytosol (cytoplasm), allowing yeast to distinguish between export and import pathways (Feldmann, 2012; Bernstein & Toth, 2012). Large pores are created by a complex molecular machinery that spans the double membrane and controls the flow of macromolecules inside and outside of the nucleus. The NPC also contributes to the short-term anchoring of activated genes or DNA damage that is difficult to repair through homologous recombination (Taddei & Gasser, 2012).

The nuclear envelope (NE) includes various chromatin anchorage sites, such as the spindle pole body (SPB), and distinct protein elements of the inner nuclear membrane that bind heterochromatin, ribosomal DNA (rDNA), or various forms of DNA damage (Taddei & Gasser, 2012). The inner and outer nuclear membranes form a double membrane bilayer that envelops the nucleoplasm, dividing it from the cytoplasm (Feldmann, 2012).

Chromosomes and the nucleosomal fibers they contain can be viewed as the fundamental components of the nucleus' structural framework (Taddei & Gasser, 2012). Saccharomyces chromosomes have a single linear double-stranded DNA strand with a few repeating sequences that are primarily brought on by the ribosomal RNA encoding process. Introns only appear in 5% of sequences (Monroy Salazar et al., 2016). It has 16 chromosomes and undergoes closed mitosis. Nuclear pores interact with chromatin, mRNA, and transport factors at the nuclear periphery to promote effective gene expression, whereas centromeres, telomeres, and silent chromatin are grouped and anchored far from pores (Blumenberg, 2019).

The nucleolus is the subnuclear compartment that is most visible and well-known (Blumenberg, 2019; Taddei & Gasser, 2012). It is a structure with a crescent shape that borders the nuclear envelope and takes up about one-third of the space in the nucleus on the other side of the SPB (Blumenberg, 2019). It is a dense area that vanishes during mitosis and reemerges during interphase (Feldmann, 2012).

The nucleolus serves as a sort of factory for ribosome biogenesis because it is where RNA polymerase I (Pol I) transcribes ribosomal DNA (rDNA), processes rRNA transcripts, and assembles

ribosomes (Blumenberg, 2019; Oakes et al., 1998). Along with other nucleolar proteins and small nucleolar RNAs (snoRNAs) necessary for these processes, Pol I and rDNA are both found in the nucleolus (Oakes et al., 1998). The rate of cell growth has a significant impact on its morphology, most likely as a result of the cell's needs dictating how quickly ribosomes are produced (Blumenberg, 2019).

It is interesting to note that various yeast strains have various numbers of tandem repeats, ranging from 100 to 200. A 35S precursor rRNA, produced by RNA polymerase I, and a 5S rRNA, produced by RNA polymerase III, are both produced by each repeat unit, which is 9.1 kb in size (Blumenberg, 2019; Taddei & Gasser, 2012). It is believed that the production of rRNA triggers the self-driven process of nucleolus assembly. This claim is supported, in part, by studies (Taddei & Gasser, 2012) showing that RNA Pol II rather than endogenous RNA Pol I was responsible for the transcription of the rDNA repeat. This resulted in significant changes to the nucleolar structure, supporting the notion that RNA Pol I-related factors are crucial for proper nucleolar assembly (Taddei & Gasser, 2012).

One 18S rRNA and 32 ribosomal proteins make up the 40S ribonucleoprotein, which is produced by polymerase I from a single 35S transcript. Pre-RNA, on the other hand, is produced by polymerase III. Pre-RNA is enclosed in a 90S RNP following transcription. These processes are all carried out through the use of exoribonuclease and endoribonuclease enzymes. These actions result in the release of 66S particles into the nucleoplasm, where they mature before being exported to the cell's cytoplasm. Additionally, they have found that additional procedures must be completed before ribosomal subunits can help with protein synthesis (Monroy Salazar et al., 2016).

The rRNA precursor molecules are broken down by particular trimming enzymes in the nucleolus and modified at approximately more than 200 nucleotide positions, through the action of particular methylases or pseudouridine synthases. The rRNAs and ribosomal proteins undergo numerous assembly steps in this compartment as well (Feldmann, 2012).

1.2 The genus *Saccharomyces*

Many species of yeast are found in the genus *Saccharomyces*, which belongs to the family of fungi (Figure 2). The yeast cell is a saprophytic, unicellular fungi cell. Many species in this genus are thought to be crucial to the production of food. Additionally, based on DNA homology, it is possible to divide the large heterogeneous species *S. cerevisiae* into four separate species. None of the four organisms, or any other species that are very closely related to them, have been linked to human pathogens or have been demonstrated to have a negative impact on the environment. The four species of the yeast *Saccharomyces cerevisiae*, *Saccharomyces pasteurianus*, *Saccharomyces paradox*, and *Saccharomyces bayanus* mentioned here are all crucial to industry (Monroy Salazar et al., 2016).

Saccharomyces bayanus
Saccharomyces boulardii
Saccharomyces bulderi
Saccharomyces cariocanus
Saccharomyces cariocus
Saccharomyces cerevisiae
Saccharomyces chevalieri
Saccharomyces dairenensis
Saccharomyces ellipsoideus
Saccharomyces eubayanus
Saccharomyces exiguus
Saccharomyces florentinus
Saccharomyces kluyveri
Saccharomyces martiniae
Saccharomyces monacensis
Saccharomyces norbensis
Saccharomyces paradoxus
Saccharomyces pastorianus
Saccharomyces spencerorum
Saccharomyces turicensis
Saccharomyces unisporus
Saccharomyces uvarum
Saccharomyces zonatus

Figure 2: *Saccharomyces* species (Monroy Salazar et al., 2016)

1.2.1 *Saccharomyces cerevisiae*

1.2.1.1 General overview

The *Saccharomyces pastorianus* yeast, which is used to ferment lager beers at the Carlsberg brewery, was first isolated in 1883 by Emil Christian Hansen. Shortly after, *Saccharomyces cerevisiae* was also isolated, and it quickly made a name for itself as the yeast that is most frequently used to make ale beers as well as a top model for genetics and cell biology (Vanderwaeren, 2022). *S.*

cerevisiae is a real organism, and its scientific name comes from the old words for beer. Although those who enjoy artisan bread or a great microbrew would probably prefer not to think of it that way, *S. cerevisiae* is classified by scientists as a fungus or mold (Duina et al., 2014).

Additionally, it is a model for an emerging fungus. Although this species can be found in many ecosystems, it is most well-known for its use as "baker's yeast" in the conventional or commercial fermentation of bread, beer, or wine. When sold as *S. boulardii*, it has also been used as a nutritional supplement and a medication to treat diarrhea brought on by antibiotics (Pérez-Torrado & Querol, 2016).

The relatively thick cell wall that surrounds *Saccharomyces cerevisiae* cells gives them their ovoid or ellipsoidal shape (Figure 3). For the large range of diameters between 5 and 10 mm and the small diameter, the cell sizes range somewhere around 1 and 7 mm. Typically, the cell size of brewing strains is larger than that of laboratory strains. *S. cerevisiae's* average cell size grows older as well (Feldmann, 2012).

Since the cells are pigmented, colonies that have grown on the surface can be seen to have a cream color. *Saccharomyces* colonies develop quickly, maturing in almost three days. Cells can be flat, smooth, moist, glistening, or dull, and can range in color from cream to tan (Monroy Salazar et al., 2016). Because of their microscopic size and straightforward growth requirements, yeast cells are cheap and simple to grow in the lab. Without the aid of special incubators, they grow into colonies on agar plates in the lab in a matter of days. Under ideal laboratory conditions, yeast cells can divide as frequently as once every 90 minutes through a process called budding in which during mitosis a smaller, genetically identical daughter cell buds off from the mother cell. Yeast stocks can be kept fresh for years by freeze-drying them or by freezing them at 280 degrees in glycerol (Duina et al., 2014).

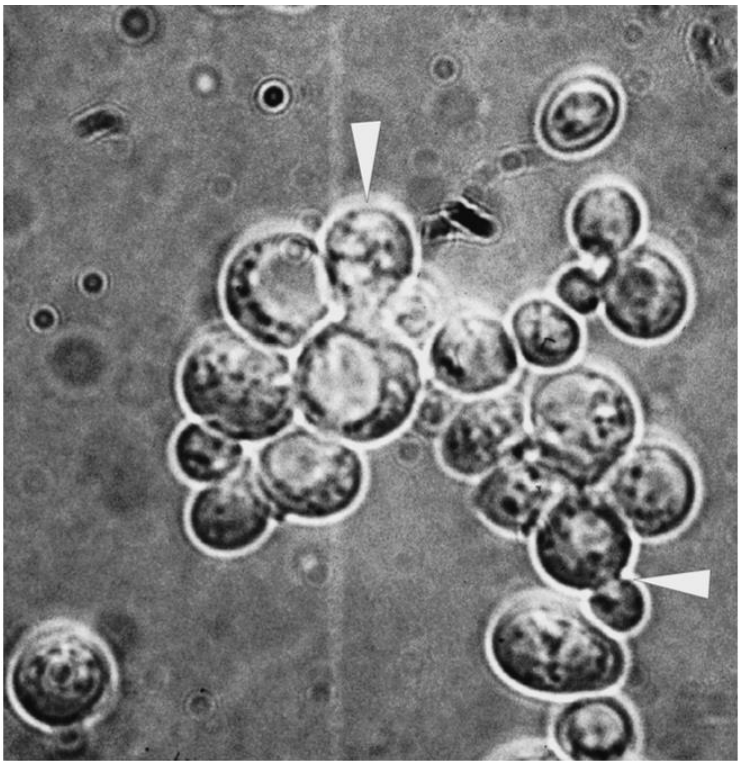

Figure 3: The Cells of *S. cerevisiae* under the microscope. The white arrows point to dividing cells

(Feldmann, 2012)

Vineyards are a natural habitat for yeasts, but they can also be found in close proximity to oak trees and in other environments. To what extent *S. cerevisiae* is a naturally occurring species or whether it is only a domesticated species is unknown (Pérez-Torrado & Querol, 2016). Recent data indicates that *Saccharomyces cerevisiae* probably originated in China and spread there, frequently being linked to humans (Heil, 2021).

Sugar is necessary for *Saccharomyces cerevisiae* to grow. They eat glucose, a form of simple sugar (Heil, 2021). In both aerobic and anaerobic environments, it breaks down sugars quickly into ethanol and carbon dioxide (Hagman, 2015). This can result in a significant amount of cellular energy (ATP) being produced when there is oxygen present. The yeast will go through anaerobic fermentation if there is no oxygen present. This process yields two adenosine triphosphate molecules as well as the byproducts of carbon dioxide and ethanol (Monroy Salazar et al., 2016).

S. cerevisiae uses sugar from grains and fruits to make fermented drinks like beer, wine, cider, and palm wine, as well as other goods like cocoa, olive oil, cheese, and bread (Heil, 2021). The rising of bread is another common use of this yeast. The dough rises and expands as a result of the production of carbon dioxide inside of it. These strains of baker's yeast are more common than ethanol-producing ones (Monroy Salazar et al., 2016).

1.2.1.2 The genome structure

Thanks to its simple genome and quick growth rate, *Saccharomyces cerevisiae* has become a useful model for a variety of studies which include biochemistry, human diseases, genetics, and even evolution. Numerous genes between humans and yeasts have been found to be homologous in humans and in fact, 2,300 yeast genes have been found to have human "orthologs," meaning that they both originated from a common ancestor. In reality, many of these genes continue to perform the same function. Nevertheless, yeast is a really effective system for better understanding biology throughout the life cycle because, unlike humans, it allows us to delete, edit, or replace genes to see what they do (Heil, 2021).

Saccharomyces cerevisiae strain S288c was cited as the first fully sequenced eukaryotic genome by the International Collaboration for the Yeast Genome Sequencing in 1996 (Vanderwaeren, 2022; Monroy Salazar et al., 2016). Following the release of the *Saccharomyces* genome sequence into the public domain, the *Saccharomyces* Genome Database has been kept up to date on a regular basis. The sequencing of the *S. cerevisiae* genome is not only a significant turning point in the development of eukaryotic biology, but it has also revealed a wealth of knowledge about different aspects of genome organization and evolution (Duina et al., 2014). *Saccharomyces* chromosomes have a single linear double-stranded DNA strand with a few repeating sequences that are primarily brought on by the ribosomal RNA encoding process and introns are found in less than 5% of sequences (Monroy Salazar et al., 2016).

The findings demonstrated that a typical *S. cerevisiae* haploid S288c cell contains about 12,156,677 base pairs of genomic DNA and carries about 6,275 genes spread across 16 chromosomes. About 5,800 are thought to be actual functional genes. It is believed to have developed from an ancestor set of 8 distinctive chromosomes following an ancient whole-genome duplication event

(Monroy Salazar et al., 2016; Vanderwaeren, 2022; Duina et al., 2014). A minimum of 31% of yeast's genes are thought to be similar to human genes (Monroy Salazar et al., 2016). There are haploid and diploid varieties of *S. cerevisiae*. Naturally occurring of this yeast strains frequently exhibit aneuploidy and polyploidy in their genomes, are genetically diverse, and are more complex (Vanderwaeren, 2022). A large number of *Saccharomyces* genes can be broadly divided into those that do not encode proteins (non-protein-coding genes) and those that can be found throughout the genome (noncoding genes) (Duina et al., 2014).

1.2.1.3 The reproduction

Heterozygous for the mating form locus *Saccharomyces cerevisiae* cells can respond to shifts in the environment's nutrient status in a number of different ways. Certain nutritional restrictions can cause cells to enter the stationary phase or change their morphology to take on a filamentous form. As an alternative, cells enter the meiotic and spore-forming pathways of development when there is no nitrogen source present and a nonfermentable carbon source is present (Neiman, 2011).

S. cerevisiae has two sexual and asexual modes of reproduction (Monroy Salazar et al., 2016). In nature, it is easy for its cells to switch between two types of mating (Duina et al., 2014). It can reproduce indefinitely as haploids (1n) and diploids (2n), where new daughter cells form through mitosis as buds that enlarge and eventually separate from the mother cell. Meiosis and the mating of two haploids to create a diploid zygote are used to switch from the haploid to the diploid phases of the life cycle. When in the presence of nutrient-deficient environmental conditions, diploids can be forced to go through meiosis and produce four haploid spores, two of each mating type (Alsammar & Delneri, 2020; Duina et al., 2014). Four haploid cells, which are encased in ascospore walls, are produced by the premeiotic S-phase division of one diploid cell and two meiotic divisions (Monroy Salazar et al., 2016). In nature, it is easy for haploid cells to switch between two types of mating *a* and *ά*, and they are both capable of producing stable haploid cells through mitosis (Duina et al., 2014; Monroy Salazar et al., 2016).

They too are able to perform sexual reproduction, in which cells of opposing mating types interact with one another through proteins called pheromones (Monroy Salazar et al., 2016). The ability of the yeasts to reproduce sexually allowed taxonomists to distinguish between the species

using the biological species concept (BSC), according to which only hybridization events between members of the same species will result in fertile hybrids (Alsammar & Delneri, 2020).

1.3 The genus *Candida*

The species of *Candida* is the fourth most widespread nosocomial bloodstream pathogen, according to the National Nosocomial Infections Surveillance System (NNISS). It is approximated that the death rate is about as much as 45 percent, possibly because of unsuitable initial antifungal treatments and inefficient testing procedures (Spampinato & Leonardi, 2013).

Candida is a fungal genus that belongs to the fungal kingdom, class *Deuteromycetes*, an auxiliary taxonomic group of fungi that contains between 150 and 200 different species, including yeast belonging to ascomycetes (sac fungi) and basidiomycetes (typical fungi) (Neppelenbroek et al., 2013). Yet, they are more than 17 of these species are known to infect humans and cause diseases when the host gets weakened or immunocompromised. More than 90% of invasive infections that have been detected in patients are caused by the most known species including *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata* and *Candida krusei*, while *Candida albicans* is the most widespread opportunistic pathogen among *Candida* species (Sardi et al., 2013; Raesi Vanani et al., 2019). Despite the fact that the relative disease cases of these pathogens vary depending on geographic area, the healthcare environment, and patient population (Spampinato & Leonardi, 2013).

Despite *Candida albicans* that predominantly cause infection in patients with diabetes and malignancy, non-Albicans Candida species such as *C. parapsilosis*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii* also trigger superficial and systemic diseases, commonly in hematology (Neppelenbroek et al., 2013). The species of *Candida* have long been associated as harmless commensals with human beings. They can be present on the mucosal surfaces of the gastrointestinal and genitourinary tracts, as well as on human skin, and become pathogenic, only when the patient's condition and immune system fail (Kabir & Ahmad, 2013).

Candidiasis is a general term referring to infections caused by Candida fungi. These diseases can affect individuals of all ages and typically occurs in the presence of risk factors for the infection that is easily detected (Pappas et al., 2018).

Candida species are the major causative causes in 50–70% of systemic fungal infectious diseases, and candidemia is the most common clinical infection, occuring in up to 15% of bloodstream infections (de Oliveira Santos et al., 2018). Candida species have been found in up to 400,000 systemic fungal infections, where they spread and settle into organs of the human body (Talapko et al., 2021; Kabir & Ahmad, 2013). This infection can cause life-threatening to people with immunosuppression including HIV patients, organ transplantation patients, and patients with cancer receiving chemotherapy and radiation treatment (Kabir & Ahmad, 2013).

1.3.1 *Candida albicans*

1.3.1.1 General overview

Candida albicans is a polymorphic yeast species that can change its external structures. It has three different forms of morphologies (Figure. 4), including blastospores, pseudohyphae, and hyphae (Talapko et al., 2021; Thompson et al., 2011). It can either grow *in vitro* or *in vivo* according to environmental conditions (Mukaremera et al., 2017). *Candida albicans* need glucose as a source of carbon and amino acids as a supply of nitrogen for their metabolism (Talapko et al., 2021).

Blastospore (yeast) is an ovoid yeast with smooth forming and round colonies, which are asexually produced by budding (Talapko et al., 2021; Berman, 2006). The newly produced bud cell starts growing from a tiny blastospore on the surface. It is frequently situated away from the birth-causing scar site. Once the growth phase is over, the cells started to split, which allows the daughter cells to divide from the parent cell (Talapko et al., 2021).

Pseudohyphae is an elongated yeast cell and the form can be varied depending on the growing conditions. The cells generally connect to a constriction at the septal site (Jacobsen et al., 2012; Thompson et al., 2011). A pseudohyphal cell colony is also sometimes referred to as a pseudo mycelium. This cell growth has been identified as a morphological growth form that shows characteristics different from blastospore and true hyphae (Talapko et al., 2021).

True hyphae is a yeast cell that has parallel walls and pores for cell contact in the septation sites. It is about 2 µm narrower compared to pseudohyphae (Talapko et al., 2021; Berman, 2006; Thompson et al., 2011).

Pseudohyphae and hyphae, in general, refer to as filamentous cells (Thompson et al., 2011). A complicated regulative network of signal paths that contain multiple transcription factors is a feature of this transition from a blastospore into a hypha. The hyphae wall contains somewhat more chitin than yeast. The wall consists of glucan, chitin, and protein, and its function is to keep the cells against difficult situations and to shield them from immunological protection by the host (Talapko et al., 2021).

The filamentation of this pathogen is driven by temperatures above 37°C, elevated CO_2 concentrations, alkali pH, serum, and also by lacking carbon and nitrogen in the existence of N-acetylglucosamine (GlcNAc) (Talapko et al., 2021). The yeast forms into hyphae when it is at ideal condition, 37°C, and neutral pH. However, when it is exposed to less-than-ideal conditions, such as low temperature and pH, it forms blastospores. Yeast's ability to change its morphology enhances survival and increases virulence (Moravek O., 2020).

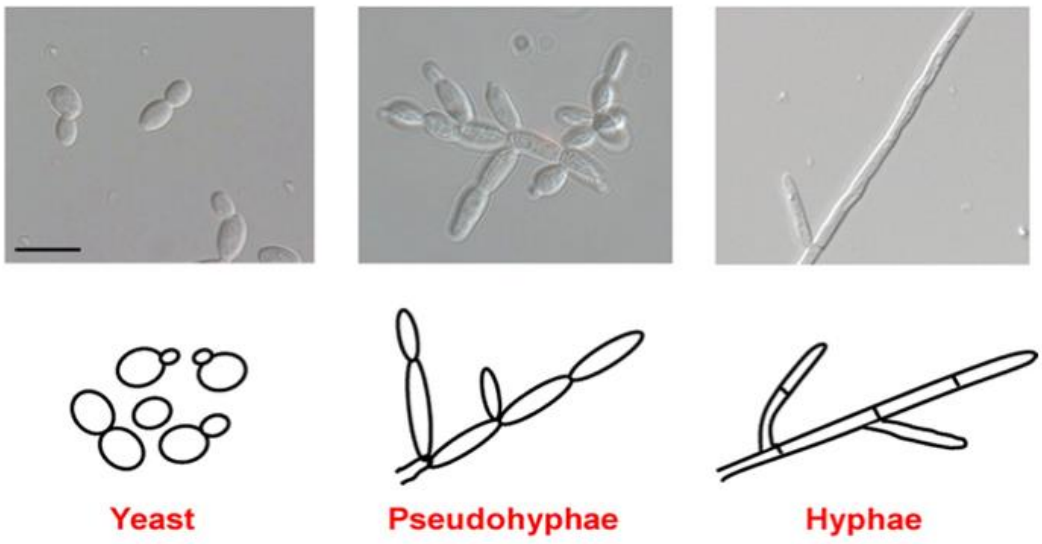

Figure 4: The main morphologies of human fungal pathogens. *C. albicans* cell images using differential interference contrast (DIC) microscopy are shown in the top image (bar = 10 µm). Every morphology is shown schematically at the bottom (Thompson et al., 2011)

1.3.1.2 Virulence factors of *Candida albicans*

Candida's virulence factors play an important role in the pathophysiology of infection incidence and progression. Some virulence factors are causing colonization or infection to start, while the rest are helping the infection to spread (Talapko et al., 2021).

Awareness of these virulence variables will be a significant tool for understanding candidiasis pathogenesis and will also assist in exploring new antifungal drugs and therapeutic goals for better medical treatments (Deorukhkar, 2014).

Polymorphism refers to the transformation of *Candida albicans* from a commensal to a pathogenic state, which is influenced by changes in the environment. The change from yeast to hyphal form is a pathogenic transformation. The hyphal form is invasive; the cells enter the host tissue through active penetration and stimulated endocytosis in this form. Host activity is required for induced endocytosis, which is mediated by hyphae invasion. Active penetration, on the other hand, is dependent on fungal activity (Talapko et al., 2021).

Another factor of virulence contributing to pathogenesis (Calderone & Fonzi, 2001 includes its capability to adhere to mucosal and epithelial cells, host recognition biomolecules (adhesins), which allow the pathogen to bind to proteins and host cells, biofilm formation, a phenotypic change to the formation of hyphae which contribute to tissue invasion, substantial thermotolerance, and hydrolytic enzyme synthesis (Calderone & Fonzi, 2001; Silva et al., 2013; Sardi et al., 2013).

Biofilm formation for *C. albicans*' pathogenicity is essential among these virulent characteristics. Its biofilm consists of heterogeneous 3-dimensional yeast and hyphal cell communities containing polysaccharides, proteins, and nuclear acids inside a self-secreted matrix of extracellular polymer substances (EPSs). *Candida albic*ans can produce biofilms and be involved in the creation of equipment-associated nosocomial infections on the surface of implantable medical devices.

Furthermore, extracellular hydrolytic enzymes are likely to play an essential role in host tissue adhesion, penetration, invasion, and destruction (Sardi et al., 2013). These hydrolytic enzymes assist the adherence to the host tissue and the break-up of a membrane of the host cell. Due to these

enzymes, it is feasible to invade mucosal membrane surfaces and blood vessels and to escape immune reactions from the host (Talapko et al., 2021).

A hypha-specific toxin, candidalysin, has been demonstrated to be essential for the development of candidiasis. Candidalysin is generated by the hyphae of the *C. albicans* and it is significantly harming the host cells that are believed to help establish systemic infection and death. Candidalysin can directly damage the epithelial membrane, producing a loss of the cytoplasmic contents by intercalation, permeability, and pores (Talapko et al., 2021).

1.3.2 *Candida parapsilosis*

While *Candida albicans* is the most widespread and invasive yeast species, its predominance has waned as the incidence of invasive infections caused by non-*Candid*a albicans species (NAC) has increased (Tóth et al., 2019). *Candida parapsilosis*, a most common NAC, has been extremely prevalent throughout the last ten years, and studies on it have also recently been raised (Trofa et al., 2008; Kročová et al., 2018).

C. parapsilosis is known as a common opportunistic yeast that can cause both superficial and invasive illnesses in humans and according to reports this pathogen is frequently the second most widely isolated *Candida* species from bloodstream infections (Souza et al., 2018; Trofa et al., 2008). Relevantly, 20–30 % of all fungal infections, especially those associated with catheters and many other medical devices, are mainly caused by *C. parapsilosis* (Zupančič et al., 2019).

C. parapsilosis has emerged as the primary cause of infection in certain European, Asian, and South American places and it is the *Candida* species that has seen the most growth since 1990. *C. parapsilosis* is the most common candidal isolate in North America, Europe, and Latin America, accounting for 15.5 %, 16.3 %, and 23.4 %, respectively. It's also the third leading cause of newborn sepsis in the United States. Immunocompromised patients, such as those with AIDS, cancer patients, and patients undergoing gastrointestinal surgeries, are more likely to develop invasive diseases. *C. parapsilosis* has a mortality rate ranging from 4% to 45 % with an average mortality rate of 28.5 %. Isolates that produce biofilms are linked to outbreaks and much greater mortality rates (Singaravelu et al., 2014).

Ashford is credited with first describing *Candida parapsilosis* in 1928 when he isolated a type of Monilia that failed to ferment mentose from feces. He coined the word *parapsilosis* to differentiate these strains from the more common *Monilia psilosis*, which was eventually renamed *Candida albicans*. *C. parasilosis* was then thought to be nonpathogenic until it was linked to a fatal case of endocarditis in a narcotic user in 1940 (Weems, 1992).

Though *C. parapsilosis* is considered a harmless microflora on human skin, it is also isolated from lesions of nails and skin (Gácser et al., 2005). In nature, *C. parapsilos*is has a broader spread and has been isolated from a number of nonhuman sources such as water, planta, soil, air, and hospital environment (Weems, 1992; Atalay et al., 2021; Sabino et al., 2011). According to McGinley *et al*., the most often isolated yeast from the subungual space among healthy volunteers was found to be *C. parapsilosis*. The species has also been studied in hospitalized patients in a number of investigations (Weems, 1992).

1.3.2.1 Virulence factors of *Candida parapsilosis*

The virulence factors of *C. parapsilosis*, which lead to candida diseases include the ability to adhesion to the host surface and a wide range of biological prosthetic surfaces, the ability to transit morphology between yeast and filamentous growth, the release of extracellular hydrolytic enzymes, the ability to build biofilms on implanted medical devices and lipid metabolisms.

Candida infection is characterized by adhesion to host surfaces, which is essential for early colonization. *Candida* can potentially build biofilms on the surfaces of medical devices. The profile of cell wall proteins and the physicochemical features of the cell surface have both been implicated in the influence of adhesion (Silva et al., 2012). The fact that *C. parapsilosis* biofilms are resistant to antifungal therapy and host immunological responses, as well as being linked to higher mortality, emphasizes their clinical significance (Chow et al., 2012).

1.4 Transcriptomic analysis

1.4.1 Gene regulation in Yeast

All living things depend on the regulation of gene expression, but microorganisms require it more than other types of organisms because they must be able to respond quickly to environmental changes. This may entail acclimating to various carbon sources or the need to use distinct metabolic pathways to get past a nutrient-restrictive circumstance. For developmental programs to be realized in higher eukaryotes and microorganisms, regulation is additionally necessary (Lang-Hinrichs, 1995).

Even presently, higher eukaryotes regulate their genes in response to environmental signals. But the interactions between cells within the organism that direct development result in a further layer of regulation. Despite the fact that prokaryotes and eukaryotes share some fundamental similarities in gene transcription. For example, the transcription process is started by RNA polymerase binding to the gene's promoter upstream of the gene, and multicellular eukaryotes regulate cell differentiation through more intricate and exact temporal and spatial regulation of gene expression. Compared to prokaryotes, multicellular eukaryotes have a genome that is much larger and divided into more chromosomes with more complex sequences. The genes of many eukaryotic species share sequences with those of other plants and animals (Phillips, 2008).

According to Thompson et al., 2013, Mary-Claire King and Allan Wilson suggested that this diversity is caused by a process known as gene regulation, which controls the timing, location, and mode of gene expression as proteins. Even though the specifics are still poorly understood, a wide range of species, such as bacteria, fungi, flies, and mammals, have confirmed the central role of gene regulation in evolution after four decades. Recently, it has been proposed that the role of gene regulation in the evolution of many different species has been significantly impacted by the duplication of genes and, in some cases, whole genomes (Thompson et al., 2013).

Based on the article of Guelzim et al., 2002, approximately 6,000 yeast genes have been identified, and genetic and biochemical studies have revealed that 124 of these genes encode regulating proteins that can control the expression of particular genes. Through 837 connections, some of the 124 regulatory genes regulate the transcription of a group of 367 non-regulatory genes. 52 of

the 124 regulatory genes interact with other regulatory genes or with themselves through an additional 72 links (Guelzim et al., 2002).

The diversity of Ascomycota fungi makes them particularly well-suited for the study of genetics and evolution. Since many of their genomes have already been sequenced, they include *C. albicans* and various yeast species. The task of determining how their genomes have evolved over time is also made easier by the fact that they have small genomes. During aerobic growth, this particular group of fungi utilizes glucose in a variety of ways as a source of carbon. The majority, including *C. albicans*, produce energy through respiratory processes called oxidative phosphorylation. Only a few, like the yeast *S. cerevisiae* and *S. pombe*, which are frequently used as models for other organisms, prefer to ferment glucose even when oxygen is present (Thompson et al., 2013).

Two kinds of mutations might help the network dynamics changes. The mutation affects processes like transcription and translation and, as a result, molds regulatory networks. These mutations alter when and how much transcription and translation occur. Conversely, the affinity of a protein for DNA or another protein can be altered by mutations, which can also alter protein activity and cause modifications in gene expression downstream (Rajeshkannan et al., 2022).

Regulation typically begins with the cell receiving an outside signal. This might be a result of hunger, stress, a particular sugar, or a particular hormone (pheromone). An internal second messenger must receive the signal. One or more genes may be affected by a cascade of reactions, which will eventually result in a response in gene expression and interaction in a coordinated manner (Lang-Hinrichs, 1995).

In particular, there are two levels of control over gene expression. The first method of controlling transcription is by regulating the quantity of mRNA that can be generated from a given gene. Post-transcriptional occurrences, which contribute to the second level of control, regulate the translation of mRNA into proteins. Post-translational modifications can influence the activity of a protein even after it has been produced (Phillips, 2008).

There are numerous levels of control over eukaryotic gene regulation. An intricate and complicated network of gene expression is supported at the transcriptional level by transcription factor (TF) binding, chromatin structure modifications, and multiple activators (Xiao & Segal, 2009).

1.4.2 Transcriptome

A transcriptome is the entire set of transcripts present in a cell or the total of all RNAs, which include messenger RNA (mRNA) and non-coding RNA (ncRNA), transcribed from a specific tissue cell at the functional state or at any particular developmental stage (Zhang, 2019; Wang et al., 2009). A transcriptome is the tiny portion of the genetic code that is translated into RNA molecules in humans, which accounts for less than 5% of the genome. More complicated organisms show up to have a higher percentage of non-protein coding transcribed sequences. Each gene can produce more than one type of mRNA (Adams, 2008). It is important to note that not all RNAs are translated into proteins. Some RNAs have structural roles, such as rRNAs in the building of ribosomes, while others are transporters and long non-coding RNAs do not result in the production of proteins but they have the potential to play a part in human illnesses like cancer, and other diseases (Blumenberg, 2019). The transcriptome can be affected by both internal and external factors, in contrast to the genome, which basically holds values that do not change often. Thus, the transcriptome acts as a dynamic connection between the genome and the physical traits of an organism (Velculescu et al., 1997).

Transcriptome research could uncover gene structure, and the functional components of the genome, advance the understanding of any particular biological processes, and mechanisms, and comprehend development and disease (Zhang, 2019; Wang et al., 2009). Cataloging all types of transcripts, that include mRNAs, non-coding RNAs, and small RNAs, is one of the main goals of transcriptomics. The study contributes to the understanding of the transcriptional gene structure, including the start sites, 5′ and 3′ ends, splicing patterns, and different post-transcriptional modifications. It also helps to determine how each transcript's expression levels change over the course of development and under various environmental conditions (Wang et al., 2009). To estimate how much gene activity or expression is occurring in a cell, the number of transcripts can be counted (Adams, 2008). Understanding the steps of cell division or embryonic development is made easier thanks to the transcriptomes of stem cells. Transcriptome analysis is a fantastic resource for finding therapeutic goals due to its extremely wide approach (Blumenberg, 2019).

Transcriptome sequencing also referred to as RNA-Seq is a newly developed technology that determines the transcriptome in provided samples and encourages and advances transcriptome research, by putting RNA-derived materials to high-throughput sequencing analysis (Zhang, 2019).

Many omics techniques, including transcriptomics, proteomics, and metabolomics, were developed following the sequencing of the human genome. The original and most popular technology is transcriptomics. In transcriptomics, the transcriptome is studied (Zhang, 2019). Transcriptomics is most frequently used to study mRNAs or coding transcripts. It also gives significant information about the structure of the non-coding RNAs, such as rRNA, tRNA, lncRNA, siRNA, and more (Blumenberg, 2019).

1.4.3 Transcription factors

A complex system that directs the genome expression is formed by transcription factors (TFs). It recognizes particular DNA sequences that regulate chromatin and the process of transcription. Although there is a great deal of interest in discovering how transcription factors (TFs) regulate gene expression, it is still unclear how TFs' exact genomic binding regions are determined and how TF binding eventually connects to transcriptional regulation (Lambert et al., 2018).

A transcription factor is a term that has previously been used to refer to any protein that plays a role in transcription and even has the ability to modify the levels of gene expression. However, the term is now only used to refer to proteins that are able to bind DNA in a manner that is specific to a given sequence and control transcription (Lambert et al., 2018). TF binds to specific DNA binding sites in gene regulation areas and regulates the transcription of those genes (Latchman, 1993). DNA binding sites can be preferred by TFs by 1,000 fold or more than other sequences. The capacity of transcription factors (TFs) to bind to specific DNA binding sites is frequently taken as a sign of their capacity to control transcription because TFs can act by blocking the DNA-binding site of other proteins (Lambert et al., 2018).

Based on the specific protein structures that they use to regulate DNA binding, which is frequently necessary, these transcription factors are grouped into families (Latchman, 1993). The main eukaryotic transcription factor (TF) families, including basic helix-loop-helix (bHLH), C2H2-zinc finger (ZF), basic leucine zipper (bZIP), homeodomain, and nuclear hormone receptor (NHR). They were first identified in 1980 (Lambert et al., 2018).

There are two fundamental aspects that make up the TF function. First, one has to be able to identify and bind brief, distinct DNA sequences found in regulatory regions. Second, has to attract or bind proteins that take part in transcriptional regulation. As a result, changes to binding sites, binding partners, and expression patterns are the main drivers of TF evolution (Mitsis et al., 2020).

1.4.4 RNA, transcription, and translation process

Ribonucleic acid (RNA) is a nucleic acid that exists in most living things as well as viruses. It is composed of nucleotides, which are made up of ribose sugars joined to nitrogenous bases and phosphate groups (Wang & Farhana, 2023). They resemble DNA quite a bit in structure but are shorter and usually single-stranded (Flatt, 2019). There are four nitrogenous bases such as cytosine, adenine, guanine, and uracil (Wang & Farhana, 2023).

RNA can be broadly classified into two types. One contains the instructions for making proteins or coding RNA, also known as messenger RNA (mRNA) and the other is non-coding RNA (ncRNA). Following the length of the RNA or its intended use, the ncRNA could be divided into a number of different types. There are short ncRNAs (20–30 not) with microRNAs (miRNAs), and small interfering (siRNAs), the small ncRNAs (~200 not) with transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA), and last one is long ncRNAs (>200 not) with ribosomal RNA (rRNA), enhancer RNA (eRNA) and long intergenic ncRNAs (lincRNAs) (Latchman, 1993).

Transcription is a process that RNA is synthesized from a segment of DNA by RNA polymerase. All forms of RNA transcription in prokaryotes are initiated by the same RNA polymerase. Different from that, in eukaryotes there are various types of RNA polymerase which is in charge of producing a particular type of RNA. For example, rRNA is produced by RNA polymerase I, mRNA by RNA polymerase II, and, tRNA by RNA polymerase III. First, the DNA promoter section is bound by the enzyme RNA polymerase to begin transcription. The DNA double helix splits into a strand that acts as a template and a non-coding strand. After that, to create a 5'-3' RNA strand with complementary nucleotides during transcription, an RNA polymerase makes use of the 3'-5' DNA template strand. With the exception of uracil replacing thymine, the newly synthesized RNA strand, and the non-coding DNA strand are almost exactly the same. Every RNA polymerase in eukaryotes

has a different mechanism for stopping the process of transcription (Wang & Farhana, 2023). The first phase of gene expression involves the transcription process. As a result, creates a primary RNA transcript from a specific gene's DNA. As such, it serves as an important first stage in the expression of genes, with numerous post-transcriptional processes like RNA splicing and translation coming after. These eventually result in the creation of a useful protein (Latchman, 1993).

Through translation, RNA's main job is to produce proteins. Genetic information is carried by RNA, and ribosomes translate it into a variety of proteins, which are very useful and required for the functioning of cells (Wang & Farhana, 2023). There are three major RNA subtypes that straight contribute to the production of proteins. They are messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) (Wang & Farhana, 2023; Flatt, 2019). mRNA has an important function to transmit messages from DNA, which regulate all cell activity. The gene responsible for producing a particular protein is turned on whenever a cell needs it to be produced, and transcription is then used to produce the necessary mRNA. To regulate the synthesis of the protein, the mRNA then engages in interactions with ribosomes and other components of the machinery of the cell. This process is referred as translation. Proteins are only produced when necessary because mRNA is comparatively unstable and not lasting in the cell, especially in prokaryotic cells. tRNAs typically only have 70–90 nucleotides. They deliver the proper amino acid to the ribosome, where protein synthesis takes place. The correct amino acid can be added to the polypeptide chain being synthesized thanks to the pair base between the tRNA and mRNA (Flatt, 2019). There are many additional functions of RNA, including editing, and gene controlling, which are controlled by small regulatory RNA such as microRNA, small interfering RNA, and small nuclear RNA (Wang & Farhana, 2023).

1.4.5 Extraction of RNA

Any molecular method must begin with nucleic acid extraction, which is essential for recovering viral nucleic acid and removing unknown components that interfere with afterward molecular applications (Akello et al. 2023). For evaluating gene expression at the mRNA level in every cell organism, the determination of steady-state levels of RNA is very important (Kurar et al., 2009). For reliable and insightful results, it is essential to isolate high-quality RNA that accurately reflects the *in vivo* transcriptional individuals of cells under all of the growth conditions (Uppuluri et al., 2007). RNA of high quality is required for researching gene expression. The quantity, as well as

the quality of the input RNA, have an impact on how accurately gene expression is assessed. The overall success of RNA-based analyses depends on the purity and integrity of the RNA. The entire efficacy of RNA-based analyses depends extensively on the purity and integrity of the RNA. Beginning with low-quality RNA could affect the outcomes of downstream processing, which are frequently challenging, take so much time, and are very costly (Tavares et al., 2011).

It is crucial to select the best RNA extraction technique. Contaminants like RNases, proteins, polysaccharides, and genomic DNA can degrade the quality of RNA and decrease the effectiveness of the amplification. Since the properties of the cell wall of fungi vary depending on their species, RNA extraction is especially important for these cells. The procedures need to be created to suit every individual situation. In comparison to DNA, RNA is also much more unstable and frequently changing (Rodríguez et al., 2018).

Several conditions must be met throughout the RNA extraction process to guarantee proper total RNA quality. For example, the final preparation must be clear of any phenol, alcohol, enzyme inhibitors, protein, and DNA, that might interfere with subsequent reactions as well as any proteins, genomic DNA, or enzyme inhibitors. Additionally, to preserve integrity according to suitable storage conditions of use, the extracted RNA must not contain any nucleases (Tavares et al., 2011).

For some techniques like RNA-Seq and qRT-PCR-based transcriptomics, high-quality RNA extraction is very essential. Quick and sterilized homogenization methods are required because single-stranded RNA is easily degraded by RNases from environmental sources. The freezing process at -80°C, adding lysis buffer, and briefly bead-beating the sample have all been demonstrated to improve the quantity and quality of RNA extraction (Hohl, 2016).

For the purpose of producing high-molecular-weight RNA, numerous methods for RNA extraction have been widely stated, particularly those used in laboratories (Andreu et al., 1988). Especially, for yeast cells, which contain an extensive amount of polysaccharides and proteins. To isolate RNA, its dense cell wall must first be destroyed. It is not very easy, and it becomes very challenging. Therefore, it is crucial to extract RNA from them with the highest quality possible (Remziye, 2012).

The quantities of RNA are now frequently measured for gene expression research using real-time PCR (qPCR) and microarray assays. Obtaining extremely reproducible using these techniques results for all organisms, including *S. cerevisiae*, depends on the quality of the initial RNA, which should have an optical density (OD) at 260 to 280 nm (A260/280) ratio between 1.9 and 2.3 (Remziye, 2012).

For advanced RNA-based techniques like microarray, RNA sequencing, and real-time reverse transcription-PCR (qRT-PCR), it is necessary to prepare RNA quickly and simply with little exposure to unwanted conditions. This ensures that the RNA is of high quality and can be obtained in sufficient quantities without degrading (Lee et al., 2019).

For RNA extraction, there are three main methods that are widely used. They are organic extraction, particularly phenol-guanidine isothiocyanate (GITC)-based solutions; spin column technology based on silica membrane; and paramagnetic particle technology. The GITC-based solution is one of the most commonly employed techniques. Yet, proteins, other elements in cells, salts, ethanol, and other organic solvents usually contaminate the RNA samples obtained by this method (Tavares et al., 2011). While widely used, the well-known phenol method is obviously too complicated and costly (Andreu et al., 1988). These techniques also call for safety measures (such as the use of fume hoods), which extend the process. In addition, the use of liquid-liquid extraction results in the unfinished separation of phases and raised contamination with DNA (Tavares et al., 2011). On the contrary, it has been reported that alkali and salt solutions can be used as extracting agents to make obtaining lower-quality crude RNA preparations easier (Andreu et al., 1988). Spin column technology based on silica membrane, and paramagnetic particle technology do not call for the use of harmful organic solvents. They produce total intact RNA with minimal protein and other material contamination. And they are comparatively simple, effective, and inexpensive. These techniques, nevertheless, frequently cause significant amounts of genomic DNA contamination (Tavares et al., 2011).

Since yeast cells were incredibly high in polysaccharides and proteins, it was important to isolate RNA from them with the highest possible quality (Remziye, 2012). Oral bacteria grown in a planktonic state, like oral streptococci, have been subject to a number of RNA extraction and purification techniques. Different from planktonic cells, the presence of an extracellular polysaccharide matrix also makes it difficult to isolate RNA from microorganisms within biofilms.

This extracellular polysaccharide matrix may obstruct the extraction and purification of the nucleic acids, particularly with the removal of genomic DNA. It is challenging to isolate and purify RNA from tissues rich in polysaccharides, such as those found in plants and fruits. These polymeric compounds bind to nucleic acids, prevent RNA from being translated, and obstruct DNAse activity. For this reason, a technique for isolating and purifying RNA that consistently produces good-quality RNA that comes from planktonic cells and biofilms in quantities large enough for functional genomic-based experiments like real-time PCR would be helpful (Cury et al., 2008). Minimum yield loss while purifying nucleic acid is required for accurate quantification of RNA and DNA from environmental samples (Lloyd et al., 2010).

For various yeast RNA extraction techniques and different commercial kits, the procedures are needed to break open the cells. To break the incredibly strong yeast cell walls, some of these techniques use hot acid phenol, shear forces, or lysing enzymes (Remziye, 2012). The most popular methods involve enzymatically disrupting the wall with zymolyase or lyticase. The latent risk of rehydrating samples and activating RNases exists regardless of the technique employed (Rodríguez et al., 2018). Following the release of the cell material, RNA is efficiently and effectively separated from other cell components with high quantity and quality (Remziye, 2012). Based on Andreu et al., 1988, RNA can be extracted from yeasts at room temperature using an ammonia solution. By using the fractional precipitation method to recover the obtained RNA, it is possible to get the yields at purity levels that are acceptable (Andreu et al., 1988). It is typically crucial to use chemiluminescent, radioactively labeled nucleotides or amino acids for accurate measurements of RNA yield from the transcription as well as to study RNA function throughout the translation. To determine the most common types of yeast RNA, the RNAs in the sample can then be examined by horizontal gel electrophoresis (Deutch & Marshall, 2008).

There are some of the most recent ways to extract RNA from the yeast *Saccharomyces cerevisiae* such as a water base technique, RNAsnap^{TM}, acid hot-phenol extraction, and one-step hot formamide extraction. Using those techniques, RNAs can be successfully extracted from *S. cerevisiae* effectively. However, other yeast species with mycelial formation with hyphae, walls of cells that are thick, and carbohydrate capsule structures frequently find it difficult to collect high-quality RNA (Lee et al., 2019).

1.5 Polymerase Chain Reaction (PCR)

1.5.1 Introduction of PCR

PCR also known as "Molecular Phototyping", is a technique that enables the amplification of nucleotide sequences and amplifies small and specifically targeted segments of DNA to generate millions of copies of a particular gene fragment from a complex DNA pool (Rajalakshmi, 2017; Ferrini, 2021; Garibyan & Avashia, 2013). It is a simple, but sensitive enzymatic assay, that can be carried out using source DNA from a wide range of cells and organisms, such as skin, hair, blood, saliva, and microorganism. For PCR to produce enough copies of the DNA to be examined using standard laboratory procedures, only very small amounts of DNA are required (Garibyan & Avashia, 2013).

Kary Mullis first invented PCR using *Taq* polymerase in 1983. He and Michael Smith shared the Nobel Prize in 1993 for their contributions to the field (Rajalakshmi, 2017; Ferrini, 2021). PCR has been consistently and steadily enhanced. It has changed many things in a variety of fields, including the medical field, genetic engineering, and the analysis of forensic evidence (Ferrini, 2021). It made it possible to specifically detect and produce significant amounts of DNA for the first time (Garibyan & Avashia, 2013). It is without a doubt regarded as one of the most significant scientific breakthroughs of the 20th century (Ferrini, 2021).

1.5.2 Required components of PCR

Each PCR assay must require some common components for the process, including thermo cyclers, template DNA, nucleotides, two primers, DNA polymerase, and PCR buffers (Garibyan & Avashia, 2013; Rajalakshmi, 2017).

- **Thermocyclers:** 0.2–0.5 ml volume thermo cyclers are used to conduct PCR reactions. To reach the necessary temperature, it alternately heats and cools the reaction tubes (Ferrini, 2021; Rajalakshmi, 2017; Garibyan & Avashia, 2013).
- **Template DNA:** is composed of the DNA that will be amplified. The segment is a tiny piece of a massive complex mixture that contains a particular genome's DNA. This DNA can come

from any source, including plasmid DNA, cDNA, and genomic DNA (gDNA). The precise part that you want to amplify is present in the DNA template (Ghannam & Varacallo, 2023).

- **Nucleotides:** the four bases that make up DNA, are adenine (A), thymine (T), cytosine (C), and guanine (G) (Ferrini, 2021; Rajalakshmi, 2017; Garibyan & Avashia, 2013).
- **Two primers:** are short artificial pieces of single-stranded DNA fragments with a maximum length of 50 nucleotides (roughly 15-30 bases). DNA polymerases need primers to serve as an extension point that tells them where to start the amplification process. Additionally, for each complementary strand of DNA, there needs to be a forward and a reverse primer (Ghannam & Varacallo, 2023).
- **DNA polymerase:** A DNA polymerase capable of operating at high temperatures is required for all PCR reactions. Commonly used Taq polymerase can amplify templates up to 5 kb and incorporate nucleotides at a rate of 60 bases per second at 70 °C, making it suitable for standard PCR without the need for special conditions.
- **PCR Buffers:** the PCR buffer makes sure that the PCR reaction is conducted under ideal conditions. Magnesium chloride ($MgCl_2$), tris-HCl, and potassium chloride (KCl) are the three main ingredients of PCR buffers. Other PCR buffers utilized include DMSO, PEG 6000, and glycerol formamide (Ferrini, 2021; Rajalakshmi, 2017; Garibyan & Avashia, 2013).

The components listed above are combined in a test tube or 96-well plate and then put in a machine that enables repeated cycles of DNA amplification to take place in three simple steps. The test tubes or plates containing the PCR reaction mixture are put into the thermal cycler's thermal block, which has holes in it (Garibyan & Avashia, 2013).

1.5.3 The principle of PCR

The fundamental idea behind PCR is the replication of a particular nucleic acid molecule as a template through primer extension in order to create double-stranded DNA. Exact temperature cycle control is another essential reaction requirement in addition to heat-resistant DNA polymerase. The three steps below are primarily involved in PCR technology (Figure 5) (Wang et al., 2023).

Denaturation: The reaction solution containing the template DNA is heated to 95 °C in the first step of PCR. The hydrogen bonds between the two DNA strands are quickly broken at high temperatures, causing the two strands to separate.

Annealing or hybridization: The reaction mixture is subsequently cooled for a further 30 to 1 minute. The ideal temperature for annealing typically ranges between 50 and 65 °C depending on the length and sequence of the primers. Each new set of primers requires careful optimization. This temperature would allow the two DNA strands to reunite. However, most don't because the mixture contains an abundance of primers that bind to the template DNA at particular and complementary spots. Hydrogen bonds will form between the primers and the template DNA after the annealing process is finished. The polymerase is now prepared to continue the DNA sequence.

Extension or elongation: The temperature used for this step is 72 °C. It is also known as the elongation temperature, at which the complementary strand is synthesized. Deoxyribonucleoside triphosphates present in the mixture are used by Taq polymerase to catalyze replication by binding to primed single-stranded DNAs. The initial double-stranded DNA molecules are transformed into more than 130 million new double-stranded molecules by reversing the cycle 30 times. Now that the newly created DNA is the same as the template strand, it will be used as such in the subsequent PCR cycles (Ghannam & Varacallo, 2023; Garibyan & Avashia, 2013; Ferrini, 2021; Yu et al., 2017; Kadri, 2020; Rajalakshmi, 2017).

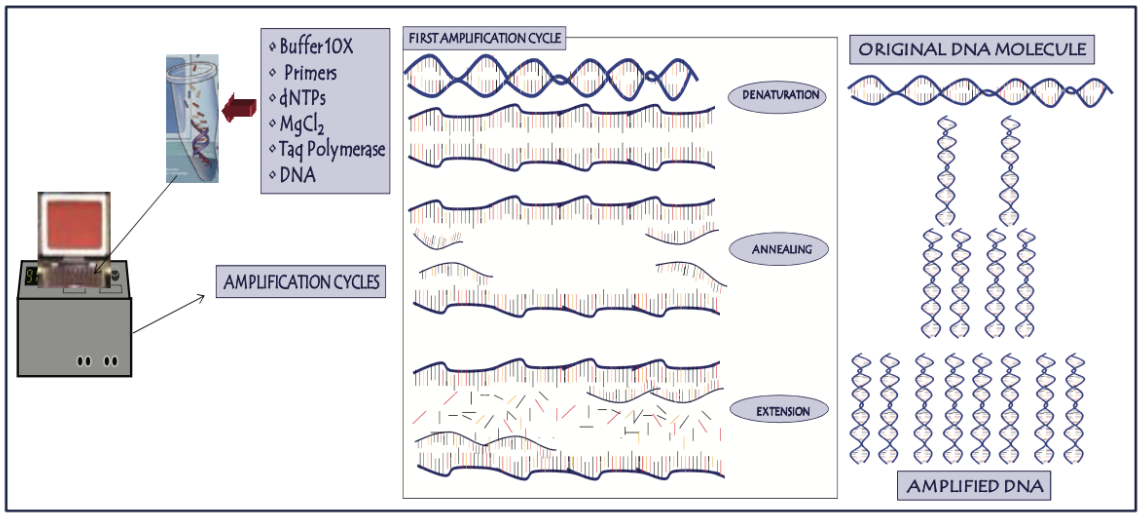

Figure 5: Required components and amplification cycles in PCR (Hernandez-Rodriguez & Gomez, 2012)

The PCR reaction takes about two hours to complete. This is dependent on the particular reaction conditions (Figure. 6). The length of the PCR reaction is primarily influenced by four variables: the temperature of the template during heating and cooling, the time it takes for the template and PCR tube to reach temperature equilibrium, the extension period, and the number of cycles (Wang et al., 2023).

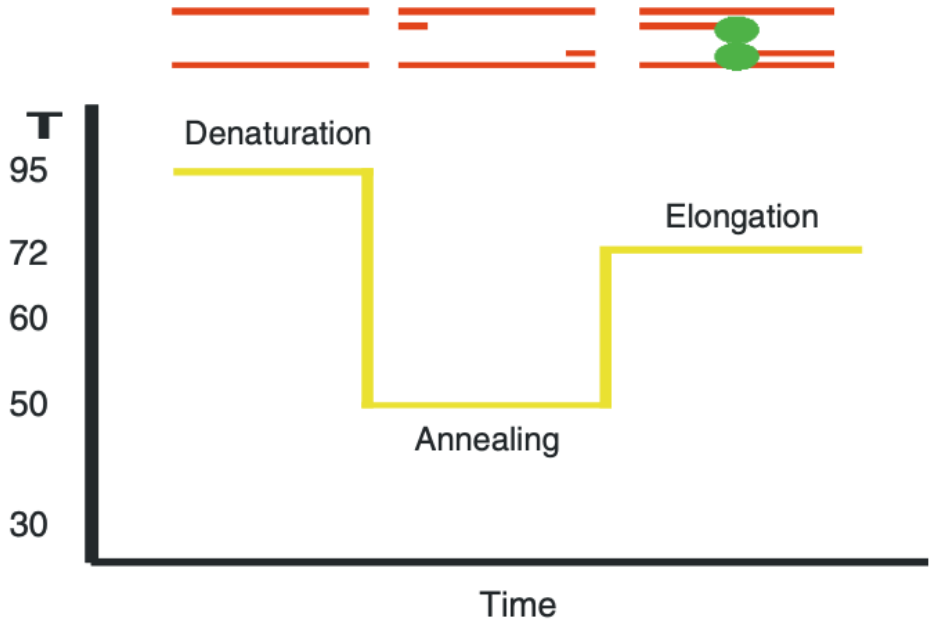

Figure 6: Temperature cycle in PCR: (1) the temperature is increased to approximately 95 °C in order to melt the double-stranded DNA (2) the temperature is decreased to allow the primers to anneal (3) the temperature is set to 72 °C in order to allow the polymerase to extend the primers (Kubista et al., 2006)

1.5.4 Different types of PCR

1.5.4.1 Generations of PCR

The first generation of PCR or the original PCR is capable of amplifying traces of DNA using an ordinary PCR machine, particularly different samples and analyzes the PCR products using agarose gel electrophoresis. PCR amplification can produce enough copies of the DNA sequences, whereas only a few DNA sequences were required for extraction (Wang et al., 2023).

Real-time PCR (qPCR) is referred to as the second generation of quantitative PCR. This technology is utilized extensively in the medical industry, including in gene therapy, DNA and RNA detection, and the diagnosis of genetic diseases (Wang et al., 2023).

The third generation is referred to as Digital PCR (dPCR), which is especially well suited for nucleic acid analysis in a complex sample with high sensitivity (Wang et al., 2023).

Modifications to the conventional PCR procedure have recently been developed. The goal is to improve efficiency and specificity and also to accomplish the high amount of the amplification of specific molecules from scientific interest (Hernandez-Rodriguez & Gomez, 2012).

1.5.4.2 Real-time PCR (qPCR)

Real-time PCR (qPCR), a PCR technological advancement that can produce quantitative results, has recently grown in significance in most medical fields. Unlike traditional PCR, which only shows qualitative results, this new technique enables corresponding the reaction and the results in a more rapid and precise manner. The quantification of DNA and RNA fragments has undergone a revolution thanks to the possibility of qPCR technology. These nucleic acids can be precisely quantified with greater reproducibility using this technique. It offers a sensitive approach for the precise quantification of each species, which may be crucial for the identification of pathogens and genetic disorders (Valones et al., 2009).

qPCR is a method that combines amplification and detection into one step by continuously gathering data during the PCR process as it takes place. This is accomplished by using several kinds of fluorescent chemistries that relate the concentration of the PCR product to the intensity of the fluorescence (Wong & Medrano, 2005). There are two popular techniques for using a fluorescence system to locate and measure the product. They are non-specific fluorescent DNA dyes and labeled probes using fluorescence (Hernandez-Rodriguez & Gomez, 2012; Garibyan & Avashia, 2013; Kralik & Ricchi, 2017). Although both of these methods are used to detect pathogens and were developed concurrently, probe-based chemistry is more common. This is because it is more susceptible to detecting non-specific PCR products, such as primer dimers, and has higher specificity, which is caused by the extra probe (Kralik & Ricchi, 2017).

A thermocycler along with an optical system to record fluorescence is needed for real-time PCR. And also a computer with software that can record the data and conduct the reaction's final evaluation is required. A signal is produced by the emission of fluorescence. It grows directly in proportion to the quantity of PCR products. The quantity of amplified products is represented by the fluorescence values that are measured and recorded throughout every cycle (Valones et al., 2009). At any given time the level of DNA present in the sample is indicated by the intensity of the fluorescent signal. The fluorescence is too low in the initial cycles to be distinguished from the background. Nevertheless, the quantity of template DNA molecules initially present in the sample corresponds to the point where the fluorescence intensity rises above the detectable level, called the quantification cycle (Kralik & Ricchi, 2017).

SYBR® Green and TaqMan® are the fluorescent materials that are utilized. Among those, the most common dye for dsDNA binding-specific is SYBR Green I. When it isn't bound to dsDNA, its fluorescence is invisible. Compared to ethidium bromide, which is the most popular dsDNA binder in traditional PCR, it has a hundred times higher affinity for binding to DNA. In addition, it is less expensive than a probe because it can be used with different pairs of different primers (Valones et al., 2009).

1.5.4.3 Reverse transcription PCR (RT-PCR)

For semiquantitative analysis, reverse transcription PCR (RT-PCR) is a commonly employed technique in biomedical studies, which includes also studies on nanotoxicology. It is a relatively easy and inexpensive method to determine the level of expression of target genes (Mo et al., 2012).

Reverse transcriptase (RT) was used in the PCR's design to generate cDNA, which was then used to amplify RNA sequences, particularly mRNA. Reverse transcriptase enzymes, DNA polymerases expressed by RNA-containing retroviruses, are used in RT-PCR to produce a complementary DNA (cDNA) from mRNA, which serves as the starting material for the DNA amplified (Hernandez-Rodriguez & Gomez, 2012; Ghannam & Varacallo, 2023). Traditional PCR techniques combined with cDNA can be used to qualitatively analyze gene expression using primer sequences for genes of interest (Ghannam & Varacallo, 2023). It has been helpful for both diagnosing RNA viruses and assessing antibiotic therapy. Due to the obtained cDNA's ability to preserve the original RNA sequence, it has also been utilized for researching gene expression *in vitro* (Hernandez-

Rodriguez & Gomez, 2012). Although they are two different techniques, real-time PCR (qPCR) and reverse-transcription PCR (RT-PCR) are frequently mixed up. Nowadays, qPCR and RT-PCR are frequently used together, allowing one to quantitatively determine the relative change in the expression of genes throughout various samples (Ghannam & Varacallo, 2023).

1.5.4.4 Reverse transcription-quantitative PCR (RT-qPCR)

Both reverse transcription (RT)-qPCR and real-time quantitative PCR (qPCR) are effective techniques for discovering how many copies of each gene are present in a sample. These techniques are the foundations for molecular ecological research and can be used to extract nucleic acids from environmental samples. They also provide useful information about *in situ* microbial activity. Post-extraction purification of nucleic acids from various environments is necessary because qPCR and RT-qPCR are sensitive to very small concentrations of inhibitors (Lloyd et al., 2010). The first and frequently most important step in carrying out RT-PCR and real-time PCR is obtaining high-quality whole RNA. RNase is very difficult to inactivate, so RNA is easily broken down. To stop RNA from degrading, several safety measures must be performed. For example, always dress in a lab coat, frequent gloves changes, be tiny on the bench, use sterile equipment and RNAse-free aqueous solutions, and do not talk when going through the RNA extraction (Mo et al., 2012).

Real-time PCR can be used in conjunction with reverse transcription PCR (Valones et al., 2009). RT-qPCR is increasingly being used in modern clinical diagnostic assays and has established itself as the gold standard for the identification and measurement of RNA targets. In addition to being more informative than qualitative data, the quantitative results produced by this technology also make assay standardization and quality control easier (Bustin & Mueller, 2005). It provides data that goes beyond simple DNA detection by showing the percentage of a particular gene or DNA in the sample. While the PCR product is being produced, it enables simultaneous real-time detection and quantification of it (Garibyan & Avashia, 2013).

Reverse transcription-quantitative PCR (RT-qPCR) is a competent PCR method used for the precise measurement of mRNA levels. This technique needs a repeatable and well-established method for RNA extraction and purification. In medical fields, RT-qPCR frequently measures mRNA levels by observing the amplicon synthesis of PCR products using the fluorescence dye SYBR Green I. This

type of fluorescence dye is specifically attached to double-stranded DNA in the minor groove (Cury et al., 2008). Compared to conventional RNA quantification techniques such as RNA microarrays, nuclease protection assays, and Northern blotting, RT-qPCR is a more sensitive method. It can be used for measuring gene expression as a substitute for measuring protein expression. Although more recent technologies like Sanger and new sequencing techniques and modern PCR techniques such as digital PCR are the same sensitive, they are more costly or call for complex bioinformatic analysis (Browne et al., 2020).

1.5.4.5 Multiplex PCR

Multiplex PCR is another PCR technology that has been developed to solve these limitations while also enhancing the diagnostic potential of PCR. The use of multiple pairs of primers in a multiplex PCR reaction allows for the amplification of multiple target sequences. While maintaining the usefulness of the tests, it has the potential to result in significant time and effort savings in the lab (Elnifro et al., 2000). It is able to identify various pathogens in a single sample. Exonic and intronic sequences in particular genes can also be found using this technique, as can the dosage of a gene (Hernandez-Rodriguez & Gomez, 2012).

Multiplex PCR has been effectively employed in numerous nucleic acid testing fields since its inception. For example, RNA detection, quantitative analysis, polymorphism analysis, as well as gene deletion analysis. This method has been demonstrated to be an effective way to identify viruses, bacteria, fungi, and/or parasites during the investigation of infectious diseases (Elnifro et al., 2000).

1.5.4.6 Nested PCR

Nested PCR (nPCR) is still widely used in labs as an effective method for increasing sensitivity and specificity, especially in settings with limited resources (Shatleh-Rantisi et al., 2020). Since the use of two sets of primers and a dual amplification process, this PCR is more sensitive because it can detect small amounts of the target. The initial set of primers enables the initial amplification. A second PCR is conducted on the result of this one using the second set of primers. The second PCR's primers are specific to an internal amplified sequence from the first PCR. As a result, the second PCR product is used to confirm the specificity of the first (Hernandez-Rodriguez & Gomez, 2012).

2 THE AIM OF THE WORK

The main goal of this thesis was to isolate RNA from different species of yeasts (*Saccharomyces cerevisiae*, *Candida albicans*, and *Candida parapsilosis*) in sufficient quantity and quality for further use. The yeast cells contain very large amounts of polysaccharides and proteins, which makes isolating RNA from them very challenging. To find the optimal method, two different methods for RNA isolation were utilized and compared by testing three different yeast species cells. The selected techniques are the conventional methods TRIzol reagent-phenol chloroform extraction and the Quick-RNA^{TM} Viral Kit from Zymo Research Corp. The obtained RNA was further analyzed by agarose gel electrophoresis and its concentration was determined spectrophotometrically.

3 EXPERIMENTAL SECTION

3.1 Materials and equipment

3.1.1 Cell materials

- ***Candida albicans***, from the Czech Collection of Microorganisms at Masaryk University, Brno
- ***Candida parapsilosis***, from the mycological collection of the Faculty of Medicine, Palacký University, Olomouc
- ***Saccharomyces cerevisiae***, from Dr. Hana Sychrová, The Institute of Physiology, Czech Academy of Science, Prague

3.1.2 Chemicals and solutions for RNA isolation using TRIzol reagent

- YNB medium - (Yeast Nitrogen Base without amino acids, Sigma Aldrich a US Biological) – 0,7% Yeast Nitrogen Base, 2% glucose, 20 mM sodium citrate, pH 5
- TRIzol™ Reagent – from Thermo, monophasic solution of phenol and guanidinium
- Isothiocyanate
- Sterile water
- Chloroform
- Isopropanol
- 75% ethanol

3.1.3 Chemicals and solutions for RNA isolation using Quick-RNA™ Viral Kit

- **Quick-RNA™ Viral Kit** (the commercial kit provided by **ZYMO RESEARCH CORP.**) – all solutions were included
- DNA/RNA Shield
- Viral RNA buffer
- Viral wash buffer
- DNase/RNase-free water
- 95-100% ethanol

3.1.4 Chemicals and solution for gel electrophoresis

- Agarose powder - (TOP-Bio)
- TBE buffer - (Tris-borate-EDTA) 45 mM Tris-borate, 1 mM EDTA
- Ethidium bromide – (TOP-Bio)
- Loading buffer – Bromphenol blue
- Marker 155-970 - (Top-Bio)

3.1.5 Equipment

- digital scale PK-601 (Denver Instrument)
- bunsen burner
- centrifuge Sorvall Lynx 6000 (Thermo Scientific)
- centrifuge MiniSpin (Eppendorf)
- environmental shaker - Incubator ES-20/60 (BioSan)
- instrumentation for electrophoresis, elektroforesis MP-300N (Major Science)
- spectrophotometer Nano-MD PDA UV-Vis Bio (Scinco)
- Thermocycler (Biometra)
- Vortex Heidolph Reax Top Test Tub (BioTech)
- UV gel viewer (Vilber Lourmat)
- microwave (LG)
- electrophoresis power source MP-300N (Major Science)
- micro cuvette
- Scino nano stick-S 0,5mm
- refrigerator
- freezer

3.1.6 Other materials

- 1.5ml, and 5ml microtubes
- Zymo-Spin™ IC Column
  (included in the commercial kit provided by **ZYMO RESEARCH CORP.**)
- beakers

- spoon, plastic weighing dish
- nitrile gloves
- pipettes, pipette tips
- measuring cylinder
- Erlenmeyer flask

3.2 Cell culture preparation

All three different yeast strains (*Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae*) were inoculated in three different Petri dishes with a solid medium (Sabouraud's agar) from frozen stock samples of microorganisms. The dishes were then placed in the incubator for 24 hours at 37°C.

The yeasts grew after incubation. They were cultivated in a set of YNB liquid mediums at pH 5, individually. They were left shaking for another 24 hours at 37°C in the laboratory shaker.

Following the incubation, the samples were tested for optical density (OD_{600}) using a spectrophotometer Nano-MD PDA UV-Vis Bio (Scinco) and were ready for further analysis.

3.3 RNA isolation methods

3.3.1 Phenol chloroform extraction using TRIzol reagent

TRIzol is an acid-guanidinium-phenol-based reagent. It was developed specifically to extract DNA, RNA, and proteins from a wide range of different biological samples (www.zymoresearch.com). A high pH of TRIzol can lead to the isolation of RNA and DNA together, whereas low pH manages the separation of RNA from DNA and proteins. The ratio of sample volume to TRIzol must be just right to effectively lyse a sample. The majority of RNA extraction problems are typically resolved by using more TRIzol than what the conventional method recommends.

Collecting cell pellet: 5 ml of each sample culture was spun down for 2 min (7000 g) in the centrifuge. The supernatant was discarded and only the pellet remained. 500 ml of sterile water was added to each tube to wash the pellet, vortexed, and transferred into three small 1,5 ml microtubes, respectively. The mixtures were spun down in the small centrifuge for 2 min (5000 g). The supernatant was again discarded.

Phase separation: The cell pellets of each sample was then suspended in 1 ml of Trizol reagent, mixed well, and left incubating for 5 min at RT (room temperature). 200 µl of chloroform was added, shaked vigorously by hand for 2-3 min at RT, and was centrifuged at 12 000 g for 15 min. Following the centrifugation, the mixture is separated into three layers (Figure 7), which means the RNA is separated from the DNA and proteins. RNA predominates in the upper, transparent aqueous phase. The white middle interphase contains DNA. The pink lower organic phase is proteins and lipids.

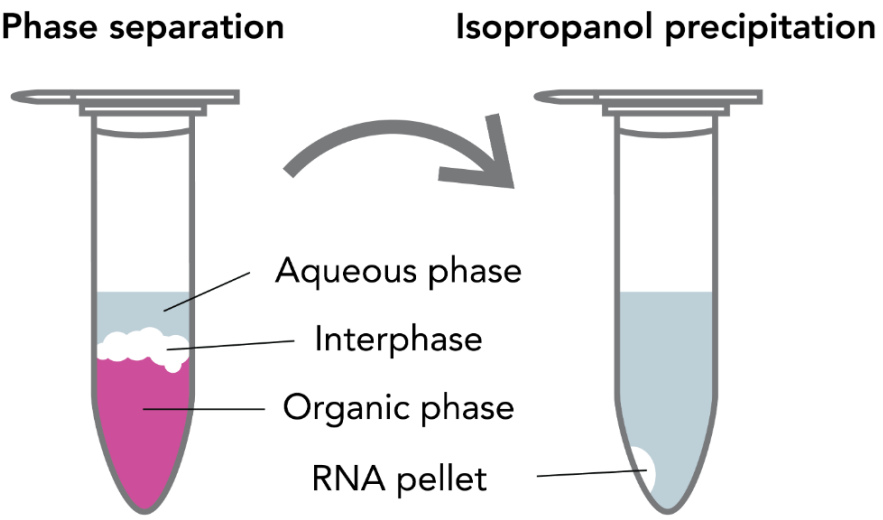

Figure 7: Three layers are shown by phenol-chloroform phase separation. RNA predominates in the upper transparent aqueous phase. The white middle interphase and the pink lower organic phase contain DNA and proteins (www.zymoresearch.eu)

RNA precipitation: The aqueous phase, which contains RNA was transferred into a fresh new microtube. The RNA was then precipitated from the aqueous phase by mixing with 500 µl of isopropanol and was left incubating for 10 min at RT followed. The mixture was subsequently

centrifuged for no more than 10 min at 12000 g. The pellet is transparent at this point and the supernatant was removed very carefully.

Washing the RNA: The RNA pellet was then washed once by adding 1 ml of 75% ethanol. The sample was briefly vortexed and centrifuged for 5 min at 7500 g. The supernatant of ethanol was discarded.

Redissolving the RNA: the pellet was left to air dry in the open tube for 30 min to an hour (or vacuum-dry for 5-10 minutes). The pellet should not be dried by centrifugation under vacuum. It is critical to avoid letting the RNA pellet completely dry as doing so will significantly reduce its solubility. The RNA pellet was resuspended in 30-50 µl of DNase/RNase-free distilled water depending on the size of the pellet and was then incubated in the thermoblock at 50-60 ^{o}C for 10-15 min.

The obtained RNA of each sample were verified by measuring the concentration of sample absorbances on the spectrophotometer Nano-MD PDA UV-Vis Bio (Scinco) at 260 and 280 nm wavelengths according to the instrument instructions. After obtaining the concentration result, the RNA of each sample was stored in the freezer at -20^{o}C for the next analysis using agarose gel electrophoresis.

3.3.2 Quick-RNA^{TM} Viral Kit

The Quick-RNA^{TM} Viral Kit is designed to quickly purify viral RNA from biological material including blood, plasma, serum, urine, saliva, cell culture media, and cellular suspensions, by keeping them in DNA/RNA Shield^{TM}. A unique buffer system is also included in the kit, which makes it easier to completely lyse viral particles for effective RNA isolation from samples containing enteroviruses and rhinoviruses. The column-bound viral RNA is cleaned and eluted after being washed. The isolated, high-quality total RNA is suitable for use in all subsequent applications, including RT-qPCR, hybridization-based, and Next-Gen sequencing (www.zymoresearch.com).

Nucleic acid stability is guaranteed by DNA/RNA Shield™ while samples are stored and transported at room temperature (4–25 ºC). The substance efficiently lyses cells, protects genetic integrity, and makes infectious agents and nucleases inactive (www.zymoresearch.com).

Note: RNA purification was performed in all steps at room temperature. The centrifugation was set up the speed to 13,000 g.

First of all, 100 µl of DNA/RNA Shield™ (2X concentrate) was added to 100 µl of each yeast species sample. The mixture was mixed well. 400 µl of Viral RNA Buffer was added to each 200 µl mixture and was mixed well. The whole mixture was transferred into Xymo-Spin™ IC Column in a collection tube and was centrifuged for 2 minutes at 13,000 g. The column was transferred into the new collection tube. 500 µl of Viral Wash Buffer was then added to the column and was centrifuged for 30 seconds. The flow-through was discarded. This step was repeated. 500 µl of 95-100% ethanol was then added to the column and got centrifuged for 1 minute to ensure the complete removal of the wash buffer. The column was carefully transferred into a new nuclease-free tube (microtube). Finally, 15 µl of DNase/RNase-Free water was added directly to the column matrix and was centrifuged again for 30 seconds.

The eluted RNA was used immediately (or can be stored in the freezer at -20ºC) to determine RNA quantity (concentration) using a spectrophotometer Nano-MD PDA UV-Vis Bio (Scinco) at a wavelength of 260 nm. The RNA was then ready to get run on a standard agarose gel of electrophoresis to examine the RNA bands.

3.4 Agarose gel electrophoretic analysis of the obtained RNA

Nucleic acid separation has been successfully accomplished using agarose gel electrophoresis. This technique separates the DNA and RNA fragments based on their size and charge (Lee et al., 2012).

Samples: The microtubes containing RNA of each sample were left thawing a few minutes at laboratory temperature after taking out of the freezer.

Preparation of the gel: 0,6 g of agarose powder was measured out into an Erlenmeyer flask to make one agarose gel. 60 ml of TBE buffer (Tris-borate-EDTA) was added into the agarose-

containing flask and was swirled to mix. The agarose suspension was heated in the Erlenmeyer flask in the microwave. The flask was taken out and thoroughly stirred the mixture every 30 seconds for 2 minutes and continued until all of the agarose has completely dissolved. The hot solution was allowed to cool down either on the work surface or under the running cool water to approximately 50-60 ^{o}C. The gel tray will warp if this is not done but be careful to not let it solidify. 4 µl of ethidium bromide (EtBr 0.5 µg/ml) was added to the cooled solution in the flask, mixed it slowly and steadily to avoid forming the bubbles. The gel tray was put inside the casting apparatus and the solution was poured into it. The comb was also placed to create wells. If larger bubbles are formed, they can be pierced with a plastic tip. The gel was allowed to be set for approximately 20 minutes at room temperature.

Note: EtBr is a chemical carcinogen that could cause significant harm to the environment and human health. Therefore, it needs to be disposed of properly following institutional regulations. For safety reasons, it is required to wear rubber gloves while handling gels containing EtBr. Although there are other dyes that can be used to stain DNA and RNA, EtBr continues to be the most widely used due to its sensitivity and affordability.

Setting up the gel apparatus: the plastic electrophoretic plate with the gel was then rotated in the electrophoretic bath so that the sample wells were closer to the cathode, as the RNA travels in the alkaline environment to the anode. The comb was carefully pulled out of the gel (vertical pull) to avoid damage to the wells of the gel which were already set. The TBE buffer was poured into the electrophoretic bath so that the conductive connection with the electrodes could be made. The layer of the TBE buffer should cover the gel approximately 2-5 mm high. Using the same running buffer as when making the gel is essential.

Loading sample preparation: a square of parafilm was prepared by pipetting 10 µl of RNA sample (note: more than 10 ng of RNA should be pipetted into the well) and mixing with 2 µl of loading buffer (blue dye). These two drops were mixed on the parafilm and were pipetted 10 µl and loaded into the well of the gel. For the RNA weight marker, 6 µl of the molecular weight marker was mixed with 2 µl of loading buffer.

Note: Loading buffer (dye) makes the sample sink into the agarose gel. It also helps us to track how far a sample has traveled during the running of electrophoresis.

Running the gel electrophoresis: all of the samples were cautiously and slowly added into the well of the gel. The electrophoretic bath was immediately closed with the lid. The gel box's leads were connected to the power source, turned on, and ensured that both the gel box and the power source are operational. The electrophoresis was performed under the constant voltage of 130 V from the power source. When it was properly connected, the bubbles were generated on the electrodes as a product of water electrophoresis. The progress of electrophoresis is monitored by the movement of the bromphenol blue, which is a part of the decomposition buffer. The bromphenol blue starts to migrate from the wells towards the anode with the sample face within a few minutes. The electrophoresis was allowed to run for about 30-45 minutes. The bromphenol blue should travel about 2/3 of the way. In the end, the power source and cables were turned off and disconnected.

Observing separated RNA fragments: the gel was carefully removed from the electric bath and transferred to the UV transilluminator, where the gel is viewed in the transmitted UV light through a UV filter by using gloves and goggles. RNA molecules that fluoresce in UV light due to ethidium bromide being bound to them, were observed.

3.5 Spectrophotometric determination of RNA concentration

The spectrophotometer which was used in this study was a spectrophotometer a Nano-MD PDA UV-Vis Bio (Scinco). This instrument is capable of measuring cell optical density at 600 nm (OD_{600}) and determining the purity of nucleic acid by giving an automatic calculation of concentration. For measuring cell optical density, a micro cuvette was used. The blank was first measured by adding 1 ml of YNB medium, and 1 ml of each culture sample was analyzed after that. On the other hand, for determining RNA concentration, a nano cuvette (Scino nano stick-S 0,5mm) was selected by adding 3 µl of sterile water, which was used as a blank. Then, 3 µl of the obtained RNA from samples was measured afterward one by one.

The yield and purity of obtained RNA of each sample were verified by measuring the absorbance of the sample at 260 and 280 nm wavelengths according to the instrument instructions. The instrument automatically calculated the RNA purity concentration within a few seconds.

4 RESULTS AND DISCUSSION

Total RNA can be extracted using a variety of techniques, including the TRIzol methods, commercial spin kits, and traditional phenol-chloroform extraction. Ribonucleases are hydrolytic enzymes that break down RNA and cause RNA degradation. RNases are found everywhere and are hard to get rid of. It is extremely important to make sure that the workplace is RNase-free during the process of RNA isolation. To compare how successful and efficient obtaining RNA with the least amount of DNA contamination was, two different methods for RNA isolation were utilized by testing three different yeast species cells. The selected techniques are conventional methods TRIzol reagent-phenol chloroform extraction and Quick-RNA^{TM} Viral Kit.

4.1 Comparison quantity of extracted yeast RNA

4.1.1 TRIzol reagent-phenol chloroform extraction

Table 2: Results of OD_{600} and concentration of *Candida albicans*, and *Candida parapsilosis*, from sample **collection A** (RNA TRIzol extraction method)

Sample A	**(OD_{600})**	**Concentration (ng/µl)**
1. *C. albicans*	2,504	**183,65**
2. *C. albicans*	2,560	68,118
3. *C. albicans*	2,215	77,02
1. *C. parapsilosis*	1,692	45,812
2. *C. parapsilosis*	1,559	42,012
3. *C. parapsilosis*	1,409	**147,208**

Table 3: Results of OD_{600} and concentration of *Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* from sample **collection B** (RNA TRIzol extraction method)

Sample B	**(OD_{600})**	**Concentration (ng/µl)**
1. *C. albicans*	2,523	30,108
2. *C. albicans*	2,409	34,969
3. *C. albicans*	2,210	17,386
4. *C. albicans*	2,802	33,863
5. *C. albicans*	2,845	**190,970**
6. *C. albicans*	3,040	114,483
1. *C. parapsilosis*	1,790	5,426
2. *C. parapsilosis*	1,743	32,926
3. *C. parapsilosis*	1,616	27,377
4. *C. parapsilosis*	2,231	60,531
5. *C. parapsilosis*	1,886	**79,387**
6. *C. parapsilosis*	1,995	48,589
1. *S. cerevisiae*	3,450	426,73
2. *S. cerevisiae*	3,383	10,272
3. *S. cerevisiae*	3,472	**231,652**
4. *S. cerevisiae*	3,483	213,482
5. *S. cerevisiae*	3,467	89,223
6. *S. cerevisiae*	3,361	18,40

Among the six samples from collection A (three samples from *Candida albicans* and another three from *Candida parapsilosis*) by using the RNA TRIzol extraction method, *Candida albicans* obtained an RNA concentration higher than *Candida parapsilosis* (Table 2). This yeast yielded the highest concentration of 183,6 ng/µl from sample 1, while *C. parapsilosis* obtained the highest of 147,2 ng/µl from sample 3.

To ensure which yeast specie provided the most RNA by using RNA TRIzol extraction, this method was used again by running another sample from collect B. This collection had nine samples in total from *C. albicans*, *C. parapsilosis*, and *S. cerevisiae* (Table 3). As a result, the highest RNA concentration, yielded by *C. albicans* was 190,9 ng/µl from sample 5. This result is very similar to the previous result. For *C. parapsilosis*, the highest RNA concentration sample was 79,3 ng/µl from sample 5, which is twice less than the result from collection A. The last one is *S. cerevisiae*, which obtained the highest RNA concentration sample of 231,6 ng/µl from sample 3.

Among these three species, S. *cerevisiae* yielded the most amount of RNA among three species by using the RNA TRIzol extraction method.

4.1.2 Quick-RNA™ Viral Kit

Another technique called Quick-RNA Viral kit silica-based matrix was run on testing nine total samples of yeasts from collection C (Table 4). The highest RNA concentration sample of *C. parapsilosis* and *S. cerevisiae* obtained were 76,9 ng/µl and 61,9 ng/µl from samples 1 and 2, respectively. Whereas *C. albicans* obtained the highest 80,3 ng/µl from sample 1, which is higher than *C. parapsilosis* and *S. cerevisiae*.

This technique was applied once more to run yeast samples from collect D in order to confirm which yeast specie provided the most RNA through Quick-RNA Viral Kit (Table 5). There were eight samples included in this collection. According to the results, the highest RNA concentration sample that *C. albicans* obtained was 288,6 ng/µl from sample 1. This result is about three times higher than the first result. *C. parapsilosis* obtained the highest RNA concentration from sample 1, which was 155,8 ng/µl, about two times more than the collection C. *S. cerevisiae* had the highest RNA concentration from sample 3 of 330,0 ng/µl, which makes it five times more than the previous result.

We noticed that all the amount of obtained RNA was increased compared to the previous result from the same method. This increasing quantity of RNA could be from the higher amount of cell optical density (OD_{600}) from each sample and the isolation procedure was done faster compared to the first time. By using this kit, *S. cerevisiae* was considered the species that obtained the most RNA among the three species.

Table 4: Results of OD_{600} and concentration of *Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* from sample **collection C** (Quick-RNA Viral kit)

Sample C	**(OD_{600})**	**Concentration (ng/µl)**
1. *C. albicans*	2,054	**80,301**
2. *C. albicans*	2,081	73,626
3. *C. albicans*	1,746	34,010
1. *C. parapsilosis*	1,699	**76,936**
2. *C. parapsilosis*	1,612	47,108
3. *C. parapsilosis*	1,767	48,002
1. *S. cerevisiae*	1,431	56,131
2. *S. cerevisiae*	1,533	**61,958**
3. *S. cerevisiae*	1,203	49,668

Table 5: Results of OD_{600} and concentration of *Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* from sample **collection D** (Quick-RNA Viral kit)

Sample D	(OD_{600})	Concentration (ng/µl)
1. *C. albicans*	2,245	**288,674**
2. *C. albicans*	2,579	254,303
1. *C. parapsilosis*	2,007	**155,824**
2. *C. parapsilosis*	1,785	116,630
3. *C. parapsilosis*	2,236	99,503
1. *S. cerevisiae*	1,771	298,821
2. *S. cerevisiae*	2,166	216,194
3. *S. cerevisiae*	1,979	**330,061**

4.2 Evaluation of the purity of extracted yeast RNA on agarose gel

4.2.1 TRIzol reagent-phenol chloroform extraction

In order to evaluate the purity of the RNA extracted from the yeasts, all the samples were visualized in agarose gel stained with ethidium bromide. The gels were then viewed in the transmitted UV light through a UV filter. The agarose gel from sample collection A by using RNA TRIzol extraction showed two tiny bands of rRNA from each yeast sample at 1300 and 900 pb (Figure 8). Even though the bands were so faint, this result could be counted as not the best nor bad. We could at least know that the rRNA bands of both species *C. albicans* and *C. parapsilosis* were there. These thin rRNA fragments could cause by the low concentration of obtained RNA. Additionally, at the bottom of the gel lane, 1, 2, and 3 of *C. albicans* showed a wide band representing the result of yeast tRNA.

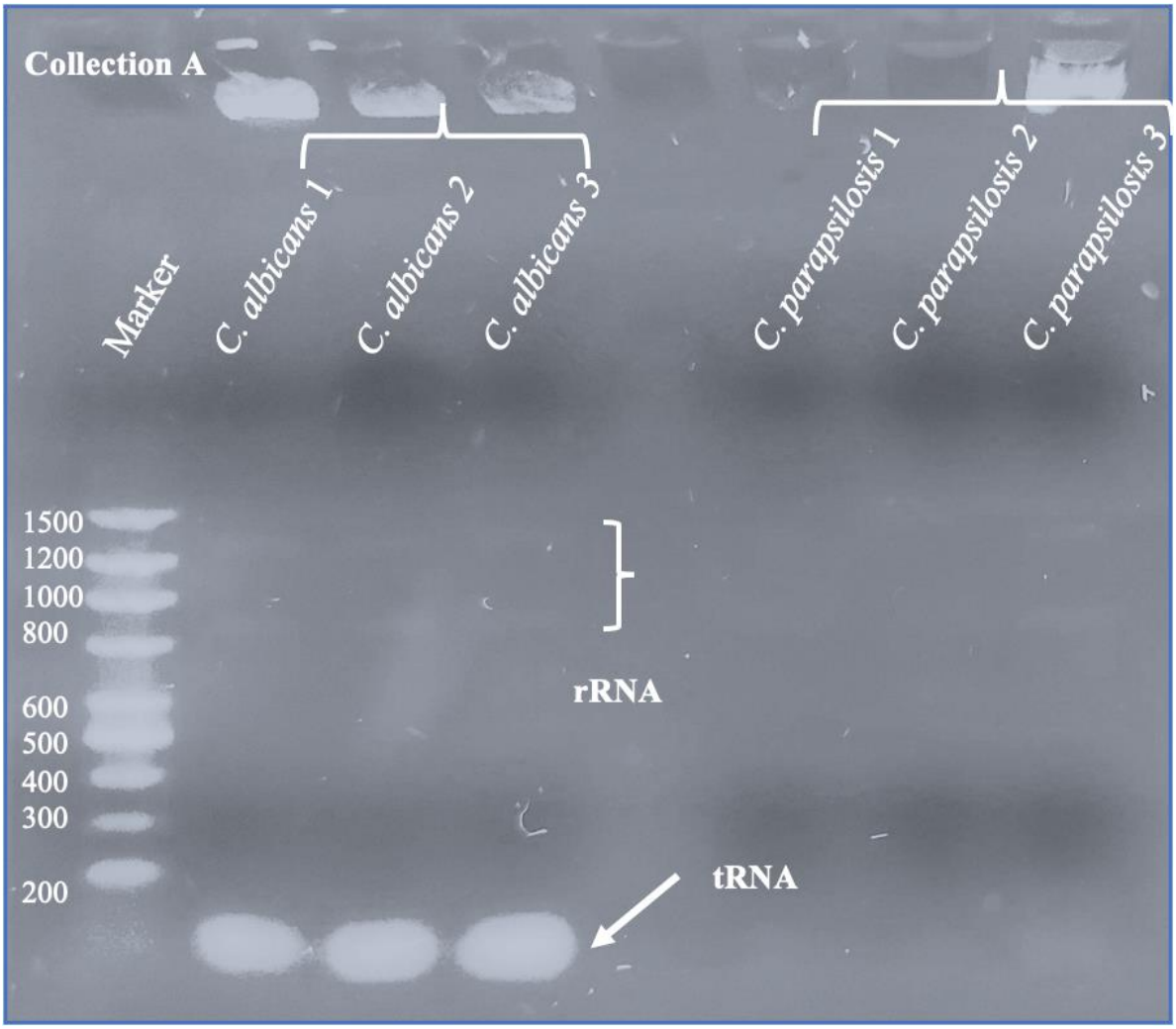

Figure 8 : Agarose gel electrophoresis of *Candida albicans*, and *Candida parapsilosis* from sample **collection A** (RNA TRIzol extraction method)

Continuing to the next agarose gel from samples in collection B, a similar result can be seen in Figure 9. If focused, we can notice two shady faint bands of rRNA between 800-1300 pb in most of *C. albicans* lanes, especially 1, 3, 4, and 5. There were also tRNA bands visible in *C. albicans* samples 4, 5, and 6, which are presented below the gel. Unfortunately, there were no RNA fragments to be observed in *C. parapsilosis* samples (Figure 9, 10).

In contrast, we observed a very small band of rRNA from *S. cerevisiae* from samples 1, 3, and 4, while the other three samples 2, 5, and 6 were all degraded (Figure 10). The RNA degradation from

most of the samples could come either from the phenol contamination while removing the aqueous layer or from RNase contamination.

To sum up, TRIzol reagent-phenol chloroform extraction is considered a suitable method for obtaining pure RNA, especially for the yeast *Candida albicans* and *Saccharomyces cerevisiae*. There was no sign of any genomic DNA contamination showing in the gels, even though the method was not used for any DNase I treatment. The two RNA bands appearing in the DNA ladder 1300 and 900 pb represent 28s and 18s ribosomal RNA, respectively.

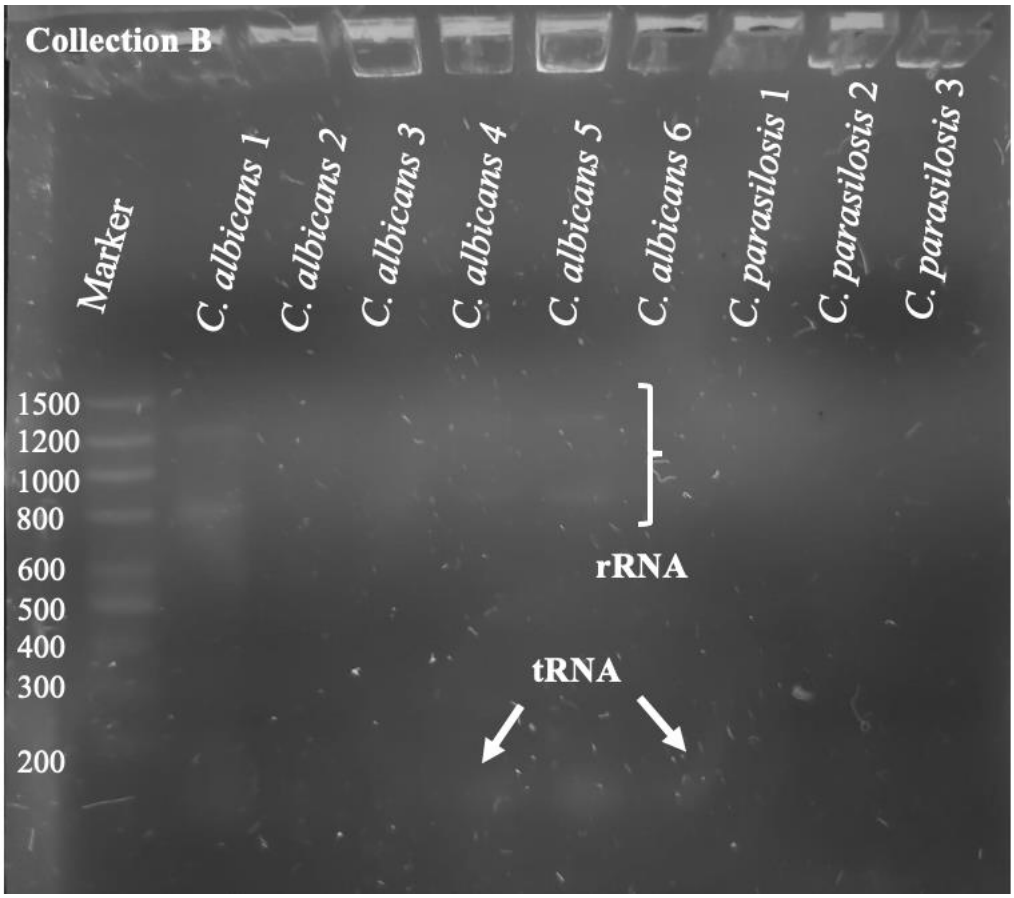

Figure 9: Agarose gel electrophoresis of *Candida albicans*, and *Candida parapsilosis* from sample **collection B** (RNA TRIzol extraction method)

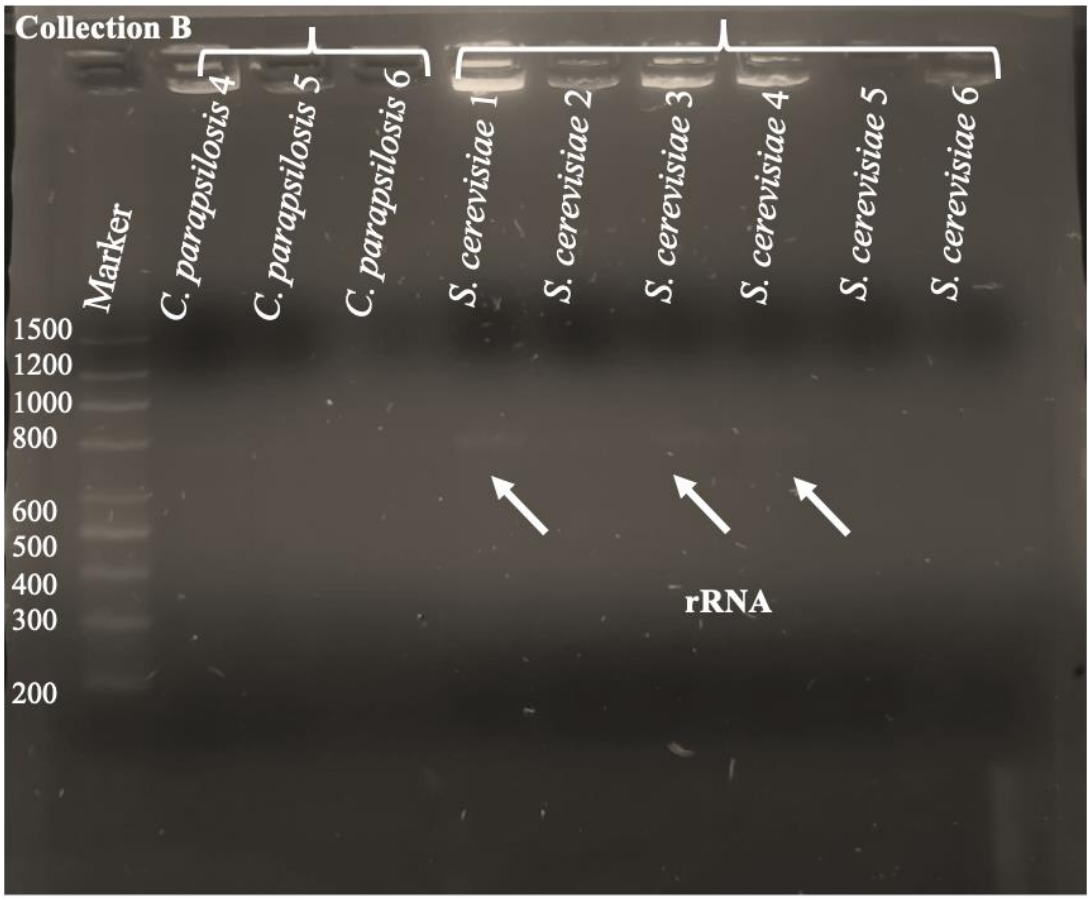

Figure 10: Agarose gel electrophoresis of *Candida parapsilosis,* and *Saccharomyces cerevisiae* from sample **collection B** (RNA TRIzol extraction method)

4.2.2 Quick-RNA™ Viral Kit

The obtained RNA by using a Quick-RNA Viral kit was evaluated by agarose gel electrophoresis also. All the rRNA bands from all samples in collection C were completely degraded (Figure 11). The causes behind this degradation could be from many reasons. One of the reasons could be from obtaining a low concentration of samples (Table 4). The other reasons could be from other contamination during isolation. Most interesting is at the bottom of the gel, wide and thick tRNA bands appeared in every yeast sample. On the other hand, DNA contamination obviously appeared on all three lanes of *S. cerevisiae*. These DNA fragments were approximately 1800-2000 pb. In contrast, there was no show of any visible genomic DNA contamination in the samples of *C. albicans* and *C. parapsilosis* even without DNase treatment.

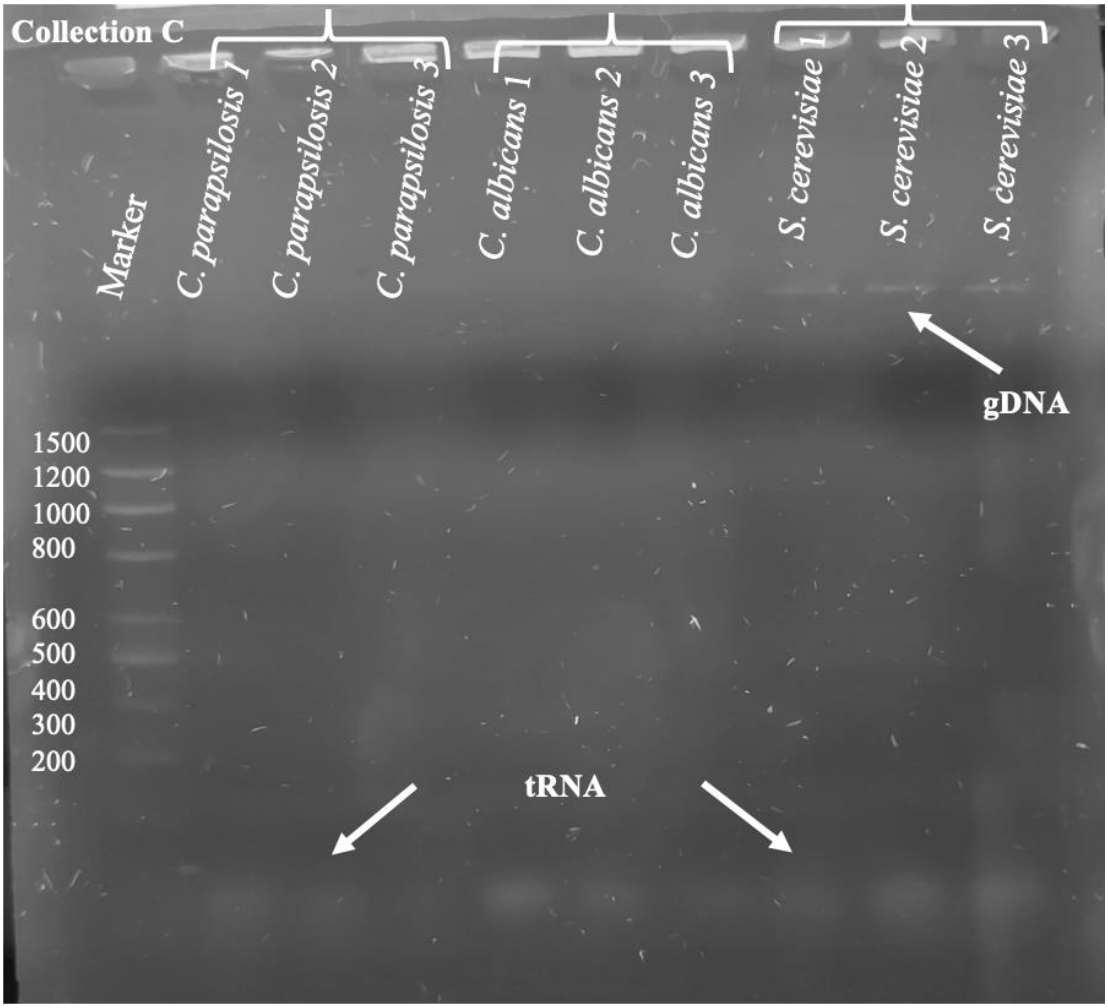

Figure 11: Agarose gel electrophoresis of *Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae* from sample **collection C** (Quick-RNA Viral kit)

Moving on to the next agarose gel (Figure 12) from all samples in collection D, which was tested by the same technique using a Quick-RNA Viral kit. It appears that we had a similar outcome as the prior gel from Figure 11. On the lanes of *S. cerevisi*ae samples, two big and clearly visible rRNA bands (1300 and 800 pb) were detected on the gel, while there weren't any rRNA fragments observed from the sample of yeast *C. albicans* and *C. parapsilosis*. The concentrations should have been big enough for the gel (Table 5). Therefore, the cause of this degradation could come from the contamination. In addition, thick tRNA bands were visible on every sample below the gel, except for the yeast *C. parapsilosis*. On the hand, there was discovered also a medium size band of gDNA on each lane of the *S. cerevisiae* samples, particularly samples 2 and 3. These bands obviously came from contamination during isolation.

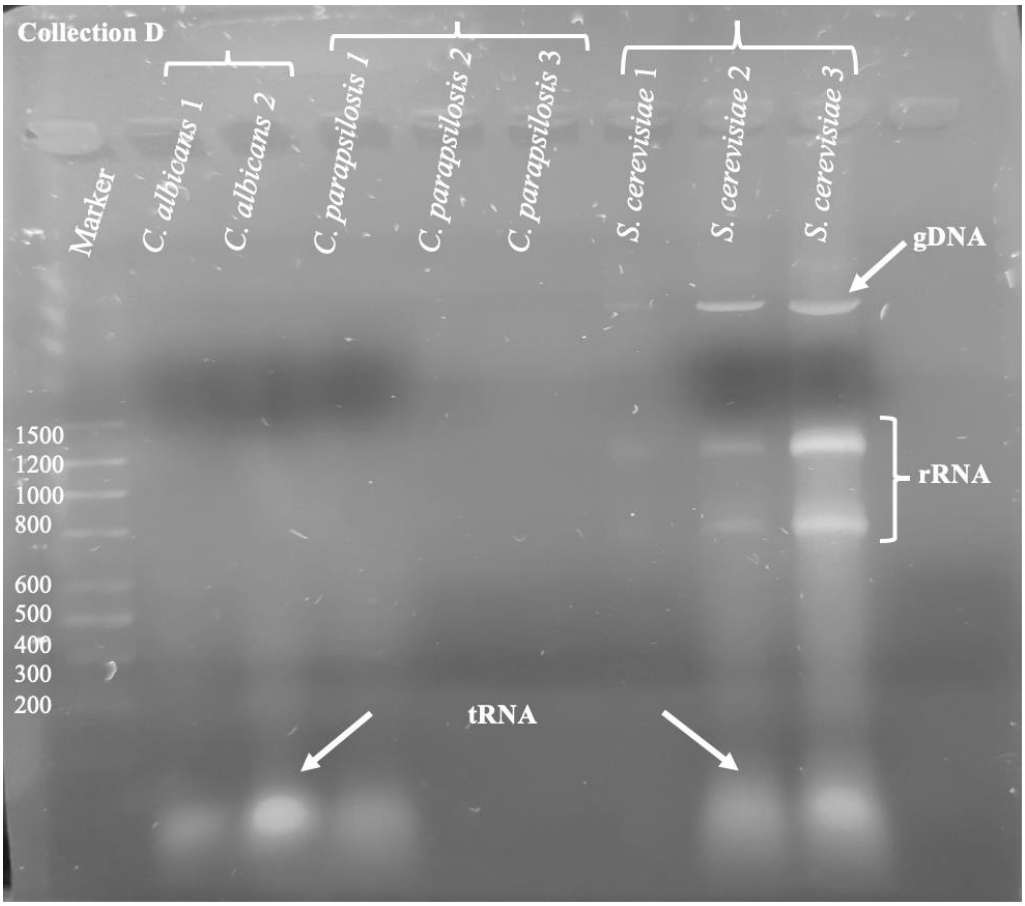

Figure 12: Agarose gel electrophoresis of *Candida albicans, Candida parapsilosis,* and *Saccharomyces cerevisiae* from sample **collection D** (Quick-RNA Viral kit)

In summary, the Quick-RNA Viral kit silica-based matrix is not regarded as an optimal technique for obtaining pure RNA and for quality purposes. Both of the results from agarose gel show that there was evidence of genomic DNA contamination from all the samples of yeast *Saccharomyces cerevisiae*. Despite the fact that there was no sign of any DNA fragments from the samples *C. albicans* and *C. parapsilosis*, it still could be possible that there was very low DNA contamination containing that was undetectable by agarose gel electrophoresis. Since DNase I treatment was not introduced in this technique, there were numerous factors could be to blame for this RNA degradation. The majority of factors could be from the contaminants during isolation. The membrane may have become clogged or contaminated with proteins or genomic DNA as a result of starting with too many samples and performing an insufficient homogenization process. Moreover, insufficient sample concentration could be one of the causes.

4.3 Evaluation of quality and quantity of obtained RNA extracted from yeasts using TRIzol reagent-phenol chloroform extraction and Quick-RNA™ Viral Kit

Table 6: Evaluation of quality and quantity of obtained RNA extracted from yeasts using TRIzol reagent-phenol chloroform extraction and Quick-RNA™ Viral Kit

Samples	RNA TRIzol Extraction			RNA Viral kit		
	$A_{260/280}$	$A_{260/230}$	C (ng/µl)	$A_{260/280}$	$A_{260/230}$	C (ng/µl)
1. *C. albicans*	1,184	0,793	**183,65**	0,932	0,502	80,301
2. *C. albicans*	1,847	0,478	68,118	1,325	0,489	**288,674**
3. *C. albicans*	1,693	0,421	77,02	1,118	0,305	254,303
1. *C. parapsilosis*	2,299	0,308	45,812	1,829	0,233	**155,824**
2. *C. parapsilosis*	2,097	0,262	42,012	1,553	0,195	116,630
3. *C. parapsilosis*	1,269	0,353	**147,208**	1,628	0,147	99,503
1 *S. cerevisiae*	2,158	1,932	**231,652**	0,820	1,209	298,821
2. *S. cerevisiae*	2,018	1,713	213,482	0,689	1,037	216,194
3. *S. cerevisiae*	2,125	1,705	89,223	0,503	1,145	**330,061**

In comparison, among nine samples from three different yeast species, TRIzol reagent-phenol chloroform extraction enables the isolation of higher quality RNA than Quick-RNA Viral Kit (Table 6). All yeast samples from *C. albicans*, *C. parapsilosis*, and *S. cerevisiae*, respectively, were qualitatively superior and provided a good A_{260}/A_{280} ratio between 1,8 – 2,1. Based on Tavares et al., 2011, A_{260}/A_{280} ratios represent the contamination level of protein or other reagents such as phenol. Therefore, A_{260}/A_{280} ratios higher than 1.8 are typically regarded as reliable signs of high-quality RNA with minimal protein contamination. An indication of low polysaccharide contamination in extracted RNA is when the A_{260}/A_{230} ratio is greater than 1.8.

Quantitatively, the Quick-RNA Viral Kit had the highest RNA yield concentration whereas the TRIzol reagent-phenol chloroform extraction had intermediate results. The yeast which yielded the highest RNA from Quick-RNA Viral Kit ranged from *Saccharomyces cerevisiae, Candida albicans,* and *Candida parapsilosis*. On the other hand, the most producible yeast from TRIzol reagent-phenol chloroform extraction was also *S. cerevisiae,* then *C. albicans*, and the lowest efficiency is *C. parapsilosis*.

These days, there hasn't been much research published particularly on yeast RNA extraction techniques as well as evaluating different commercial kits for yeasts.

According to the article of Remziye, extraction methods using TRIzol reagent and a commercial kit (High Pure RNA Isolation Kit) were compared and evaluated. The publication focused on the isolation of *Saccharomyces cerevisiae* RNA using various pretreatments with varying concentrations of yeast cells and the enzyme lyticase (Sigma). The study result suggests that using the TRIzol reagent with a high concentration of yeast cells and lyticase enzyme all play significant roles in the RNA isolation process, in terms of high yield and quality. Choosing a suitable protocol is very crucial. This research's findings show a similar impact to our study's findings.

Additionally, based on Tavares et al., six different commercial kits for RNA extraction were selected and tested using SK-N-MC cells. One of the kits was TRIzol® Plus RNA Purification System (Invitrogen). This study research claims that TRIzol® Plus RNA Purification System obtained intermediate quantitative values for both RNA yield and concentration. The results also showed that

using DNase treatment prevented DNA contamination, despite the fact that it takes a lot of time and adds significantly to the cost of these studies.

Indeed, a study by Rodríguez et al., comparing and evaluating the purity of RNA isolation using two commercial systems (the Qiagen system and the Zymo Research system) revealed that the Zymo Research system generated RNA that was of lower quality and was moderately degraded than the Qiagen system. However, it was superior at obtaining an RNA with less phenols and carbohydrate contamination and had a notably higher RNA yield. This result is somehow related and shows the same result as our finding, regarding the lower quality of RNA along with providing a high yield of Quick-RNA Viral Kit from the Zymo Research system. On the other hand, they mentioned that even though the running of the eletrophoresis method is a little complicated, the quality of RNA extract does not directly depend on its concentration or its spectrophotometry values. This part is in some ways contrary to what we observed in our study.

5 CONCLUSION

The isolation of yeast RNA has been very tricky and challenging. Especially, for the yeast cells that contain an extensive amount of polysaccharides and proteins. RNA has a short life and is easily degraded by RNases, which can be found everywhere. It is important to limit the contamination. RNA samples devoid of DNA contamination are necessary for gene expression study experiments. Moreover, due to the possibility of DNA being amplified during the PCR portion of the experiment, DNA removal is particularly essential for reversed transcription PCR. These days, there are so many RNA extraction methods available ranging from conventional to modern. Choosing a suitable and optimized technique for yeast RNA isolation is not easy.

In comparing the two methods used for RNA isolation, TRIzol reagent-phenol chloroform extraction was found to be a better technique that tested all three yeast species, especially *Candida albicans*, in terms of quality. This method delivers RNA free of genomic DNA and proteins without the use of DNase I treatment. Some researchers believe that DNase treatment may result in a loss of both RNA quantity and mRNA integrity. It could increase the number of salts and proteins in the sample and decreases how effectively cDNA is produced later (Tavares et al., 2011). At the same time, the quantity of obtained RNA by this technique is still low but acceptable compared to the Quick-RNA Viral Kit. The protocol of this method is well-established and frequently used, even inexperienced researchers can easily complete the process. TRIzol reagent acts as a main factor in qualifying the obtained RNA. It is easily used and has the ability to denature RNases. However, this reagent is expensive and made of many harmful chemicals, which is bad for humans as well as the environment. Most important, using TRIzol the sample can get contaminated by phenol during the transfer of the aqueous layer.

In terms of quantity, Quick-RNA Viral Kit yielded higher RNA concentration than TRIzol reagent-phenol chloroform extraction for all yeast species, especially for *Saccharomyces cerevisiae*. The procedure of this method is very simple, easy, and fast. There is also no use of any harsh chemicals. However, as DNase I was not treated in this method, the result showed that *S. cerevisiae* was contaminated by DNA. During the experiment, some of the pellet samples were big and got clogged to the membrane of the column, which could also result in protein and DNA contamination. In conclusion, the aim of the thesis was accomplished.

Controlling the RNA quality during isolation is very crucial. When the available amount of RNA is small, but the quantity needed is large, the method is needed to be improved. The new method could be designed by combining different techniques, which can be most optimized for the samples. Many other kits can be utilized in combination with TRIzol extraction and silica column, which can help to speed up and simplify the experiment. This could be a good option, which has yet to be investigated for the yeasts.

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